DER EINFLUSS VON GLUKOKORTIKOIDEN AUF DIE TRACHEALE CHLORIDSEKRETION

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Inhaltsverzeichnis

1. Abkürzungsverzeichnis

ABC-Transporter	
Proteinkinase B	AKT
anoctamin 1	Ano1
Adenosin 5´tri (di) phosphate	ATP/ADP
calcium-activated chloride channel	CaCC
zyklisches AMP	cAMP
zystische Fibrose	CF
cystic fibrosis transmembrane conductance regulator	CFTR
casein kinase 2	CK2
Dexamethason, dexamethasone	Dexa
Ethylendiamintetraessigsäure	EDTA
epithelialer Natriumkanal	ENaC
endoplasmatic reticulum-associated degradation pathway	ERAD
Glukokortikoide, glucocorticoids	GC
Glukokortikoidrezeptor	GR
Guanosintriphosphatase	GTPase
Horseradish peroxidase	HRP
mukoziliäre Clearance	MCC
mitochondrial ribosomal protein s18a	Mrps18a
Natrium-Kalium-Adenosintriphosphatase	Na,K-ATPase
nukleotidbindende Domäne	NBD
N-myc downregulated gene 1	NDRG1
Neural precursor cell expressed developmentally downregulated gene 4-like	NEDD4L
Natrium-Kalium-Cotransporter	NKCC1
periziliäre Flüssigkeitsschicht, periciliary liquid layer	PCL

PSD 95/SAP 90, Disc large, Zonula occludens-1	PDZ
Phosphoinositid-3-Kinase	PI3K
FYVE finger-containing phosphoinositide kinase	PIKfyve
Proteinkinase A/B/C	PK- A/B/C
cytoplasmatische regulatorische Domäne	R-Domäne
Respiratory distress syndrome	RDS
real time quantitative polymerase chain reaction	RT-qPCR
serine/threonine-protein kinase, serum and glucocorticoid-regulated kinase 1	SGK1
transmembrane domain	TMD
transmembrane member 16A	TMEM16A

2.Abbildungsverzeichnis

3. Bibliographische Beschreibung:

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4. Einleitung

Glukokortikoide (GC) haben vielfältige Einflüsse auf physiologische Regulationskaskaden. So wird durch GC u.a. der Ionentransport im respiratorischen Epithel reguliert. In distalen Atemwegen wirken sich GC stimulierend auf den epithelialen Natriumkanal (ENaC) aus. Hierdurch kommt es intrauterin zur Umstellung des initial sekretorischen Phänotyps des fetalen respiratorischen Epithels zum Überwiegen absorptiver Vorgänge. Als Folge nimmt die intraluminale Flüssigkeit innerhalb der fetalen Atemwege ab und die Diffusionsstrecke des Sauerstoffs wird verringert. Dies stellt sich als Resultat parallel ansteigender maternaler GC-Serumlevel dar und bildet somit einen bedeutenden Teil der perinatalen Transition der fetalen Lunge zum postnatal gasaustauschenden System. Vor Beginn der Transition überwiegt die Aktivität eines sekretorischen apikalen Chloridkanals, dem *cystic fibrosis conductance regulator* (CFTR). Als Gegenspieler zum ENaC gewährleistet der CFTR einen transepithelialen Flüssigkeitsstrom in das Lumen des respiratorischen Systems mit daraus resultierender Initiierung eines Proliferations- sowie Differenzierungsreizes auf das Lungengewebe. (Harding und Hooper 1996; Larson et al. 2000)

Die Lunge lässt sich hinsichtlich Funktionalität als auch Anatomie in unterschiedliche Kompartimente unterteilen. Man spricht vom gasaustauschenden sowie vom luftleitenden Kompartiment; ebenso unterscheiden sich die jeweiligen Bereiche hinsichtlich Zellverbandsstruktur und Ionenkanalbesatz. Die bisher veröffentlichten Daten zum Einfluss von GC auf pulmonale Ionentransporte bezogen sich meist auf distale Bereiche, wohingegen wenig über die Wirkung von GC auf die in den proximalen Atemwegen ablaufenden Transportprozesse bekannt ist. Im Hinblick auf die Anwendung von GC zur Induktion der Lungenreifung bei drohender Frühgeburt oder als anti-inflammatorisches Medikament stellt sich die Frage, ob und in welcher Form es zu einer Beeinflussung des proximalen pulmonalen Ionentransportes kommt. Beachtet man, dass die mukoziliäre Clearance (MCC), die Selbstreinigungsfunktion der Lunge, sehr stark von einem Gleichgewicht der periziliären Flüssigkeitsbewegungen als Folge gerichteter perimembranöser Ionentransporte abhängt, nimmt die Bedeutung der Frage nach den Auswirkungen eines gängigen Therapeutikums neben der Erlangung größeren Verständnisses für physiologische Prozesse weiter zu. Daher beschäftigt sich diese Arbeit mit den Auswirkungen von GC auf den Ionentransport des proximalen respiratorischen Epithels im Hinblick auf den CFTR und hiermit vergesellschafteter intrazellulärer Signalwege.

4.1 Der CFTR und seine Rolle im epithelialen Chloridtransport

Die Funktionalität diverser schleimhautauskleidender Epithelien ist abhängig von einem aufeinander abgestimmten Gleichgewicht absorbierender wie auch sezernierender Vorgänge. Der CFTR ist an der apikalen Plasmamembran der Epithelzelle einiger solcher Epithelien zu finden. So z.B. im Pankreas, den Schweißdrüsen, dem Dünndarm, dem Nierenepithel sowie den Atemwegen.

In den Atemwegen generiert im sekretorisch aktiven Epithel eine basolaterale Na, K-ATPase einen den ebenfalls basolateralen Na-K-2Cl-Cotransporter (NKCC) antreibenden Natriumgradienten. Die dadurch ansteigende intrazelluläre Chloridkonzentration sowie eine aufgrund des basalen Kaliumausstroms hyperpolarisierte Membran bilden die Triebkraft für den extrazellulär gerichteten Ausstrom der Chloridionen über Chloridkanäle der apikalen Membran, wie dem CFTR. Dem daraus entstehenden osmotischen Gradienten folgt Wasser transzellulär ins Lumen. (Cassin et al. 1986; Olver und Strang 1974; Barker et al. 1995a)

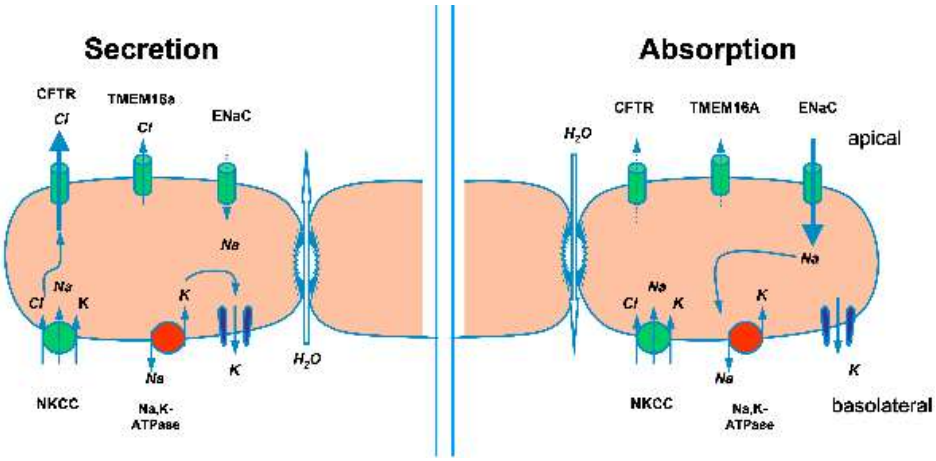

Abbildung 1: Flussdiagramm Ionenströme im Atemwegsepithel. Bei der Sekretion kommt es durch eine über einen basalen Na^+-Gradienten angetriebenen NKCC zum Ansteigen von $[Cl^-]_{iz}$ wodurch die Triebkraft für den nach intraluminal gerichteten Cl^--Efflux durch den CFTR bereitgestellt wird. Den Ionen folgt Wasser transzellulär. Kommt es aufgrund apikal vorhandener ENaC jedoch zum Na^+-Einstrom in die Zelle, kann kein Cl^--Ausstrom erfolgen und der intraluminale Ionenverlust bewirkt eine Absorption.

Im Falle einer überwiegenden Aktivität des ENaC wird die durch die Na, K-ATPase aufgebaute Triebkraft genutzt, Natriumionen über die apikale Membran in das Zellinnere zu transportieren. Dieser Prozess wirkt aufgrund des ebenfalls trans- und parazellulär folgenden Wassers absorbierend und somit reduzierend auf das intraluminale Flüssigkeitsvolumen.

4.2 Die Struktur und Regulation des CFTR

Der CFTR ist ein membrangebundener Proteinkomplex mit den Eigenschaften eines Adenosin 5´triphosphat (ATP)-abhängigen Chloridionenkanals. (Anderson et al. 1991a; Anderson et al. 1991b; Kartner et al. 1991) Kodiert durch einen Bereich auf Chromosom 7, entsteht nach posttranslationaler Modifikation das CFTR-Protein. Der funktionsfähige Kanal besteht aus zwei Untereinheiten mit je sechs kanalbildenden Transmembrandomänen (TMD), zwei Nukleotidbindungsdomänen (NBD) sowie zytoplasmatischer regulatorischer Domäne (R-Domäne) als Bindeglied zur Interaktion mit regulierenden Proteinen. Diese R-Domäne kann durch Proteinkinasen phosphoryliert und der Kanal somit aktiviert werden. Außerdem enthält der CFTR weitere Bindedomänen für Protein-Protein-Wechselwirkungen. So besteht über den C-Terminus die Möglichkeit zur Interaktion mit über die PDZ-Domäne regulierend wirkender Proteine. Hierbei handelt es sich um sequenzspezifisch proteinbindende Domänen zur Signalübertragung in der intra- als auch interzellulären Kommunikation. (Nourry et al. 2003) PDZ stellt ein Akronym aus den drei Proteinen dar, an welchen diese Domäne erstmals beschrieben werden konnte (PSD95/SAP90, Discs large, Zonula occludens-1). Aufgrund der Homologie der beiden NBDs und damit bestehender großer Ähnlichkeit zu anderen ABC-Transportern, wird der CFTR dieser Transporterfamilie zugeordnet. (Hyde et al. 1990; Deeley und Cole 1997)

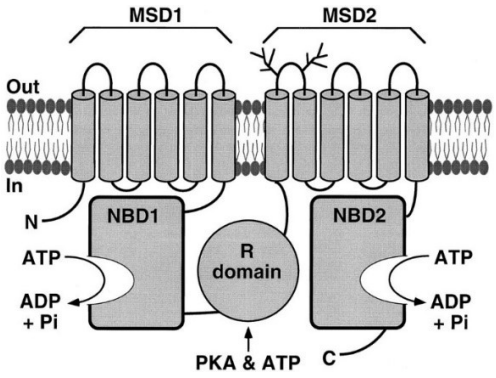

Abbildung 2: Struktur des CFTR. Der CFTR setzt sich zusammen aus zwei Untereinheiten, die jeweils aus sechs Transmembrandomänen (TMD) geformt werden. Anhängig befinden sich an jeder TMD zwei Nukleotidbindungsdomänen (NBD) sowie eine zytoplasmatische regulatorische Domäne, über die eine Steuerung der Aktivität des CFTR ermöglicht wird. Über C-terminale Strukturen bestehen weitere Interaktionsmöglichkeiten mit intrazellulären Strukturen. (Sheppard und Welsh 1999)

Der CFTR durchläuft auf dem Wege zum zellmembranständigen funktionsfähigen Protein eine komplexe posttranslationale Modifikation. (Patrick und Thomas 2012) Im Rahmen dieser Prozessierung kommt es zu hydrophoben Anlagerungen nahe des C-Terminus der NBD. Diese sind essentiell für die Reifung und Stabilität des Proteins. (Gentzsch und Riordan 2001) So gelangen lediglich 20-40% erfolgreich gefaltetes und glykosyliertes Wildtyp-Protein in heterologen Zellsystemen (HeLa-Zelllinie) an die Zelloberfläche. Bis zu 75% werden davor im ERAD System (*ER-associated degradation pathway*) abgebaut. (Ward und Kopito 1994) In CFTR-exprimierenden Zelllinien wie Calu-3 sowie T84 werden hingegen nahezu 100 % erfolgreich auf der Zellmembran exprimiert. Diese Unterschiede gehen vermutlich auf eine zwischen den Zelltypen divergierende Ausstattung an der Proteinreifung beteiligter Proteine zurück. (Varga et al. 2004)

Beim CFTR handelt es sich um einen nahezu vollständig anionselektiven Chloridkanal mit geringem Einzelkanalstrom sowie linearer Strom-Spannung-Beziehung. Die Offenwahrscheinlichkeit ist bestimmt durch ein eng abgestimmtes Netzwerk aus Proteinkinasen und Phosphatasen, welche an den vielen Konsensus-Phosphorylierungsstellen der R-Domäne und den ebenfalls dort befindlichen geladenen Aminosäuren ansetzen. (Hyde et al. 1990; Sheppard und Welsh 1999) Nach erfolgter Phosphorylierung sowie bei Anwesenheit von ATP an der NBD und dort erfolgter ATP-Hydrolyse öffnet sich der Ionenkanal. Somit stellt ATP einen essentiellen Cofaktor für die Funktionsfähigkeit des Kanals dar. Die Phosphorylierung an der R-Domäne des intrazellulären Teils des CFTR erfolgt durch die Proteinkinase A (PK-A) und die Proteinkinase C (PK-C). (Valdivieso und Santa-Coloma 2013) Die PK-A stellt dabei die bedeutendste Kinase hinsichtlich der Regulierung der Aktivität des CFTR als Chloridionenkanal dar. Die PK-C allein vermag, verglichen mit den Stromflüssen nach Aktivierung durch die PK-A, lediglich 15 % des CFTR-Stroms hervorzurufen. (Berger et al. 1993) Bei Blockierung der PK-C-abhängigen Phosphorylierung zeigt sich jedoch, dass die PK-A alleine den CFTR in heterologen Zellsystemen nicht aktivieren kann. (Jia et al. 1997) Jia et al. interpretieren daher eine konstitutive Phosphorylierung des CFTR durch die PK-C als Voraussetzung für eine durch die PK-A ausgeführte Aktivierung des CFTR-abhängigen Chloridstroms. Der Abbau des CFTR von der Zelloberfläche erfolgt durch Ubiquitinylierung mittels Ubiquitinligasen, wie der *Neural Precursor Cell Expressed, Developmentally Downregulated 4-Like* (NEDD4L), gefolgt von anschließender Degradierung im Proteasomen-Komplex. (Ward et al. 1995)

4.3 Die (Patho-) Physiologie des CFTR

Der CFTR spielt über einen osmotisch bedingten transepithelialen Flüssigkeitstransport eine Rolle in der Regulierung der Viskosität diverser Körpersekrete aus Pankreas, Schweißdrüsen, Dünndarm, Niere sowie dem respiratorischen Epithel. Je nach beteiligtem Organ hat dies Einfluss auf die Funktionalität der Verdauung, Hormonsekretion, Temperatur- und Salzhaushaltregulation sowie auf den Gasaustausch. Beim respiratorischen Epithel scheint der CFTR bereits an der Entwicklung pulmonaler Strukturen beteiligt zu sein. Die Höhe der CFTR-Expression ist entwicklungsabhängig reguliert. Im Epithel der fetalen Atemwege liegt die höchste Expressionsdichte im ersten und zweiten Trimenon der Gravidität vor, gefolgt von einem Rückgang im dritten Trimenon. (Trezise et al. 1993; Tizzano et al. 1993; Tebbutt et al. 1995; Broackes-Carter et al. 2002) In der postnatalen Lunge hingegen ist die Expression des CFTR nahezu einzig auf submuköse Drüsen der luftleitenden Atemwege beschränkt. (Engelhardt et al. 1992; Engelhardt et al. 1994) So ist die Lunge während der fetalen Entwicklung intraluminal mit aktiv durch das Lungenepithel sezernierter, vor allem chloridhaltiger Flüssigkeit gefüllt. (Jost und Policard 1949; Alcorn et al. 1977; Adamson et al. 1969) Studien lassen vermuten, dass die CFTR-Expression während der Lungenentwicklung durch eine Steigerung des intraluminalen Flüssigkeitsdrucks einen Proliferations- wie auch Differenzierungsreiz auf das pulmonale Epithel ausübt. (Harding und Hooper 1996; Larson et al. 2000) Bei fetalen Lämmern wurde gezeigt, dass eine Drainage der pulmonalen intraluminalen Flüssigkeit und damit einhergehende Aufhebung des intraluminal erzeugten Drucks eine fehlende Ausdifferenzierung des Lungenepithels sowie eine Hypoplasie der Lunge zur Folge hat. (Alcorn et al. 1977)

Kommt es jedoch durch eine intrauterine Okklusion der fetalen Trachea oder durch eine verstärkte CFTR-Expression nach viralem Gentransfer *in utero* zur Zunahme der intraluminalen Flüssigkeit, führt dies zu einer gesteigerten pulmonalen Zellproliferation sowie vermehrten Differenzierung des respiratorischen Epithels. (Paepe et al. 1998; Harrison et al. 1996) Dieses Prinzips bediente man sich im Tiermodell der kongenitalen Zwerchfellhernie, einem schwerwiegenden Krankheitsbild, welches in einer Lungenhypoplasie mit unzureichender Differenzierung des respiratorischen Epithels resultiert. Die intrauterine Überexpression des CFTR verbesserte die Alveolarisierung, möglicherweise durch Steigerung der intrauterinen Flüssigkeitssekretion und Erhöhung des intraluminalen Drucks. (Larson und Cohen 2006; Larson et al. 2000) Diese Studien legen eine zentrale Bedeutung der intrauterinen Chlorid- und Flüssigkeitssekretion und der daraus resultierenden Druckverhältnisse bei der fetalen Lungenentwicklung nahe.

Des Weiteren bildet jene Flüssigkeit zu einem großen Anteil das Fruchtwasser, welches den Feten *in utero* umgibt und als Schutz bei Bewegungen dient. Im Laufe der intrauterinen Entwicklung erfahren die Ionentransportvorgänge des Atemwegepithels, zeitgleich zum Anstieg fetaler GC-Serumlevel, eine Veränderung. Distale Alveolarepithelien werden durch eine gesteigerte ENaC-vermittelte Natriumabsorption sowie durch einen Rückgang der CFTR-vermittelten Chloridsekretion zu überwiegend Flüssigkeit absorbierenden Zellen. Diesen Mechanismus nutzt man für die Lungenreifeinduktion bei drohender Frühgeburt. Treten Störungen der pränatalen Elektrolytbewegungen auf, kann z.B. das Atemnotsyndrom des Frühgeborenen (RDS*, postnatal respiratory distress syndrome*), eine sogenannte feuchte Lunge mit eingeschränkter Diffusionskapazität aufgrund reduzierter Gasaustauschfläche sowie verlängerter Diffusionsstrecke, die Folge sein. (Kindler et al. 1993)

Auch die Selbstreinigungsfunktion der Lunge ist angewiesen auf ausgeglichene Flüssigkeits-bewegungen über das Lungenepithel. Aus den CFTR-abhängigen Transportmechanismen resultiert der Aufbau eines periziliären Flüssigkeitsfilms (PCL). (Tarran et al. 2006; Tarran et al. 2002) Dieser dient der Lunge als Partikelvektor. Der korrekte zweigeschichtete Aufbau des PCL ermöglicht es den zilientragenden Zellen des Atemwegepithels die in der obersten Schicht gelösten Partikel durch

oralwärts gerichteten Zilienschlag hinauf zu befördern. Bei Fehlregulationen kommt es aufgrund eines ineffizienten Abtransports zu chronischen Entzündungen. (Livraghi und Randell 2007) Auch Pilewski et al konnten zeigen, dass im Falle einer eingeschränkten CFTR-Funktion und einer damit verbundenen Hyperabsorption von Natriumchlorid durch den ENaC der periziliäre Salz- und Wassergehalt abnimmt und hierdurch die MCC in ihrer Funktion beeinträchtigt wird. (Pilewski und Frizzell 1999) Hinzukommend scheint Prednison schon in kleiner Dosis die Transportfähigkeit des Mukus zu reduzieren. (Oliveira-Braga et al. 2012) Auch in diesem Zusammenhang sind bisher wenige Daten über die Auswirkungen von GC verfügbar.

Defekte im CFTR-Gen äußern sich letztendlich in einer Abnahme der CFTR-Einzelkanalaktivität oder in einer verminderten Oberflächenexpression des membrangebundenen Kanals, der Ursache der zystischen Fibrose (CF), einer autosomal-rezessiven Erkrankung. (Riordan et al. 1989) Bei der häufigsten Mutation im CFTR-Gen, deltaF508, fehlt aufgrund einer Deletion von drei Nukleotiden an der Stelle 508 die Aminosäure Phenylalanin. (Riordan et al. 1989) Das Protein kann nicht korrekt gefaltet werden, weshalb es nach der Proteinqualitätskontrolle im ERAD-System durch das Proteasom abgebaut wird. (Lukacs und Verkman 2012) Aufgrund der daraus resultierenden verminderten CFTR-Aktivität kommt es zu einer erhöhten intraluminalen Sekretviskosität und daraus folgender Organfehlfunktion. (Farrell et al. 2008) Neben deltaF508 gibt es zahlreiche weitere Mutationen im CFTR-Allel wie Frameshift-, Stopp-, und Substitutionsmutationen, aus denen in der Gesamtheit eine geminderte Funktionalität resultiert. (Kerem et al. 1989) Auch durch eine Überaktivierung des CFTR können sich Pathologien entwickeln. So kommt es intestinal bei der Cholera durch bakterielle Exotoxine zu wässrigen Durchfällen. Dieses Enterotoxin sorgt über die Blockade der GTPase zu einer überschießenden Aktivität der Adenylatzyklase und daraus hervorgehend zu einer ungeregelten Zunahme an intrazellulärem cAMP. Durch diesen second messenger wird der CFTR kontinuierlich aktiviert, wodurch sich anhaltende wässrige Durchfälle einstellen, die bei unzureichendem Ausgleich des Flüssigkeitsverlustes v.a. bei Kindern rasch zum Tode führen können. (Cassel und Pfeuffer 1978)

4.4 alternative Chloridkanäle

Aufgrund von Beobachtungen, dass CF-Patienten sowie CF-knockout-Tiere nahezu keine makroskopischen Auffälligkeiten in der initialen Lungengerüstarchitektur besitzen, geht man davon aus, dass es neben dem CFTR weitere Chlorid-sezernierende Ionenkanäle im respiratorischen Epithel geben muss. Auf diesem Wege muss der intraluminale Flüssigkeitsdruck gewährleistet werden, ohne den eine suffiziente Reifung der Lunge nicht ablaufen kann. (Barker et al. 1995b; Clarke et al. 1994) So findet die Ionenkanalfamilie der *Calcium-activated chloride channel* (CaCC) im Zusammenhang mit einem epithelialen Chloridkanal Erwähnung. Der *transmembrane member 16A*, auch Anoctamin 1 genannt (TMEM16a/Ano1), als Vertreter dieser Familie, konnte in sezernierenden Epithelien, so auch dem respiratorischen Epithel, nachgewiesen werden. (Huang et al. 2009; Oh und Jung 2016) Ferner scheint es auch bei Fehlen der TMEM16A-Funktion zu einer eingeschränkten MCC zu kommen. (Ousingsawat et al. 2009; Rock et al. 2009) Mehr Beachtung gewann der TMEM16A durch die Beobachtung, dass nach einem knockout des TMEM16A bei voll funktionsfähigem CFTR eine Tracheomalazie, unvollständige Knorpelspangen sowie instabile Atemwege die Folgen waren.

Im Hinblick auf die vielfältigen Prozesse an denen der CFTR bzw. dessen korrekte Funktion gekoppelt ist, ist es das Ziel dieser Arbeit, der Frage nachzugehen, welchen Einfluss GC auf die Aktivität des CFTR ausüben und welche Regulationskaskaden z.B. im Proteinkinasenetzwerk dem unterliegen. Aus dem Verständnis der physiologischen Grundlagen dieser Transitionsregulation könnten Schlüsse zur Verbesserung der Behandlung von Lungenerkrankungen mit Schwerpunkt auf eine Fehlregulation der Flüssigkeitshomöostase gezogen werden.

4.5 Methodik – Etablierung der Methode

Etablierung einer primären Trachealzellkultur

Zur Gewinnung der primären Zellkultur erfolgten die Präparation von Rattentracheae sowie eine Kultivierung der Zellen auf kollagenbeschichteten Membraneinsätzen, sogenannten SnapWells. Die gewonnenen Zellverbände wurden für elektrophysiologische Messungen in der Ussing-Kammer genutzt. Zur Unterscheidung verschiedener Ionenkanäle wurden spezifische Aktivatoren sowie Inhibitoren verwendet. Aus parallelen Zellkulturansätzen wurde mit Hilfe von RNA-Kits mRNA gewonnen. Die Menge an mRNA konnte nach Umschreibung in cDNA mit Hilfe der RT-qPCR quantifiziert werden. Mittels Western Blot wurden Phosphorylierungszustände von betrachteten Kinasen bzw. Ligasen untersucht, um Rückschlüsse auf den Aktivierungsstatus zu ermöglichen. Die statistischen Analysen wurden mit GraphPad Prism durchgeführt. Die genauen Protokolle finden sich ausführlich in den jeweiligen beiden Publikationen. Registriernummer des Versuchsvorhabens: Landesdirektion Leipzig, T36/13 vom 21.12.2012.

Das erste Ziel meiner Arbeit bestand in der Etablierung einer verlässlichen Methode zur Gewinnung einer primären Atemwegszellkultur. Als Modell bedienten wir uns der Ratte, da Studien eine größere Ähnlichkeit in der zellulären Zusammensetzung des Atemwegsepithels zum Menschen, verglichen mit sonst geläufigen Mausmodellen, gezeigt haben. (Widdicombe et al. 2001) Zusätzlich wurde die Genauigkeit der Messungen optimiert, indem ausschließlich drei Knorpelspangen oberhalb der Carina und drei Knorpelspangen unterhalb des Larynx verwendet wurden, um eine Mischkultur zu vermeiden. Dabei bediente ich mich unterschiedlicher publizierter Präparations- und Kulturanleitungen. (Kaartinen et al. 1993; Stutts et al. 1985; Yamaya et al. 1992; Lee et al. 1984; Bukowy et al. 2011; Widdicombe 1990) Die Kultivierungsbedingungen wurden in mehreren Aspekten variiert und optimiert. So musste u.a. die Zusammensetzung beschriebener Kulturmedien verändert werden, da diese z.B. *Ethylendiamintetraacetat* (EDTA) als Mediumzusatz verwendeten. Die Anwesenheit von EDTA hemmte jedoch die Enzymaktivität bei der enzymatischen Dissoziation des Gewebes. Die verwendete DNAse benötigt Magnesiumionen als essentiellen Cofaktor, welche jedoch durch EDTA als Komplexbildner cheliert werden. Darüber hinaus stellte sich der Zellverdau über 21 h bei 4 °C auf einem Wippbrett als optimal heraus. Ohne die kontinuierliche Durchmischung des Ansatzes schien es den Enzymen nicht möglich, den Zellverband suffizient von der trachealen Matrix zu lösen. Außerdem zeigten sich die abgelösten Zellen als Aggregate und es kam zu keinem suffizienten Adhärieren der Zellen auf den Membranen. Um eben dieses Anwachsen zu optimieren, wurden die Kultivierungsmembranen mit Kollagen beschichtet, wodurch ein effizienteres Wachstum erreicht werden konnte. (Widdicombe 1990)

Elektrophysiologische Messung der Kanalaktivität

Die Messung der Elektrolytbewegungen über das Epithel erfolgte mit Hilfe der Ussing Kammer. In einem Multi-Kanal-Kammersystem konnten bis zu 8 Epithelien parallel gemessen werden. Angeschlossen an eine variable Gleichstromquelle ist es möglich, anhand der Veränderungen von Potentialdifferenzen, Widerständen und Strömen sowie über die Zugabe unterschiedlicher Aktivatoren und Inhibitoren Rückschlüsse auf die Ionentransportvorgänge des Epithels zu erzielen.

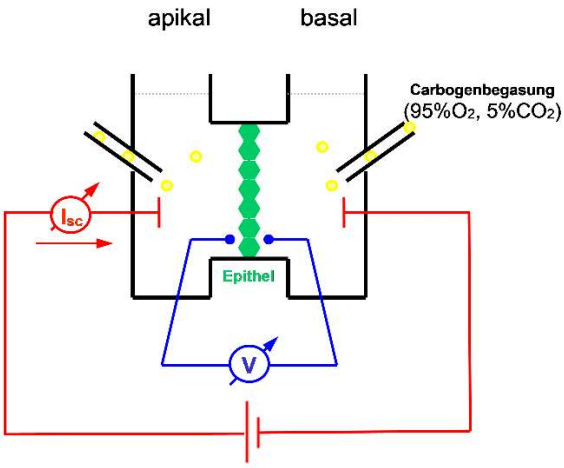

Abbildung 3: Schema der Ussing Kammer. Durch das eingespannte Epithel in apikaler-basaler Ausrichtung wird die Kompartimentierung *in vivo* nachempfunden. Über Messungen von Strom, Spannung sowie Widerständen nach Veränderungen der Elektrolytzusammensetzung oder nach Hinzugabe spezifischer Inhibitoren/Aktivatoren sowie Veränderungen der anliegenden Gleichstromquelle können Aussagen über die Ionenkanalaktivität getroffen werden.

Untersuchung der mRNA-Expression

Zur Klärung der Wirkung von GC auf die mRNA-Expression bediente ich mich der RT-qPCR. Bei diesem Verfahren wird in Abhängigkeit zur amplifizierten Menge einer zuvor aus mRNA synthetisierten cDNA mit Hilfe von SYBR-Green eine Fluoreszenzintensität induziert. Mit Hilfe einer in der Konzentration vordefinierten cDNA-Verdünnungsreihe konnte diese Fluoreszenz zur Quantifizierung der cDNA- und damit auch der mRNA-Menge genutzt werden. Als Referenzgen verwendeten wir Mrps18a, ein mitochondriales ribosomales Protein, dessen Expression, verglichen mit anderen bekannten Referenzgenen, nicht durch die experimentellen Bedingungen beeinflusst wurde. (unveröffentlichte Ergebnisse der Arbeitsgruppe) Die gemessene cDNA-Konzentration ist somit proportional zur vorher aus der Zellkultur gewonnenen mRNA.

Analyse der Proteinphosphorylierung

Entgegen des ersten Vorhabens konnten keine Messungen der CFTR-Protein-Expression mittels Western-Blot durchgeführt werden, da sich keiner der zum Zeitpunkt der Experimente kommerziell verfügbaren Antikörper als spezifisch genug herausstellte, um eine verlässliche Protein-Expressionsmessung zu gewährleisten.

Mit Hilfe von Western Blots, die den Phosphorylierungsstatus bestimmter Proteine über primäre Antikörper bekannter Phosphorylierungssequenzen messen, erfolgte die Aktivitätsermittlung einiger Proteinkinasen wie der *serum/glucocorticoid regulated kinase1* (SGK1), der Proteinkinase B (PKB/AKT) sowie der NEDD4L. Sekundäre Antikörper, konjugiert mit Meerrettich-Peroxidase (*horseradish peroxidase* – HRP) erlaubten über Chemilumineszenz eine Bestimmung der Proteinexpression bzw. des Phosphorylierungsstatus.

5. Originalpublikation

5.1

Glucocorticoids Distinctively Modulate the CFTR Channel with Possible Implications in Lung Development and Transition into Extrauterine Life

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Glucocorticoids Distinctively Modulate the CFTR Channel with Possible Implications in Lung Development and Transition into Extrauterine Life

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Abstract

During fetal development, the lung is filled with fluid that is secreted by an active Cl- transport promoting lung growth. The basolateral Na^+,K^+,2Cl^- cotransporter (NKCC1) participates in Cl^- secretion. The apical Cl^- channels responsible for secretion are unknown but studies suggest an involvement of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is developmentally regulated with a high expression in early fetal development and a decline in late gestation. Perinatal lung transition is triggered by hormones that stimulate alveolar Na^+ channels resulting in fluid absorption. Little is known on how hormones affect pulmonary Cl^- channels. Since the rise of fetal cortisol levels correlates with the decrease in fetal CFTR expression, a causal relation may be assumed. The aim of this study was to analyze the influence of glucocorticoids on pulmonary Cl^- channels. Alveolar cells from fetal and adult rats, A549 cells, bronchial Calu-3 and 16HBE14o- cells, and primary rat airway cells were studied with real-time quantitative PCR and Ussing chambers. In fetal and adult alveolar cells, glucocorticoids strongly reduced Cftr expression and channel activity, which was prevented by mifepristone. In bronchial and primary airway cells CFTR mRNA expression was also reduced, whereas channel activity was increased which was prevented by LY-294002 in Calu-3 cells. Therefore, glucocorticoids strongly reduce CFTR expression while their effect on CFTR activity depends on the physiological function of the cells. Another apical Cl^- channel, anoctamin 1 showed a glucocorticoid-induced reduction of mRNA expression in alveolar cells and an increase in bronchial cells. Furthermore, voltage-gated chloride channel 5 and anoctamine 6 mRNA expression were increased in alveolar cells. NKCC1 expression was reduced by glucocorticoids in alveolar and bronchial cells alike. The results demonstrate that glucocorticoids differentially modulate pulmonary Cl^- channels and are likely causing the decline of CFTR during late gestation in preparation for perinatal lung transition.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

During fetal development the lung is filled with fluid which is actively secreted by lung epithelial cells [1]. This lung fluid has a discrete composition consisting of a high Cl^- and low protein concentration and is the product of an active Cl^- secretion into the lumen. Vectorial Cl^- transport drives water secretion into the lung which is crucial for lung expansion and thereby for growth of the developing airways and alveoli [1]. Alterations of fluid dynamics result in developmental lung anomalies. Tracheal obstruction and accumulation of lung fluid lead to lung hyperplasia whereas drainage of lung liquid results in profound hypoplastic changes in the lung [2]. Cl^- enters the basolateral membrane across the Na^+,K^+,2Cl^- cotransporter (NKCC1) while Na^+ is actively extruded by the Na,K-ATPase. K^+ channels recycle K^+ at the basolateral side hyperpolarizing the membrane which represents the driving force for apical Cl^- extrusion. Therefore, fetal lung fluid secretion can be inhibited by blockers of NKCC like bumetanide or inhibiting the Na,K-ATPase by ouabain [3–5]. However, the transporter mediating the extrusion of Cl^- at the apical side of epithelial cells is unknown, yet evidence suggests an involvement of the cystic fibrosis transmembrane conductance regulator (CFTR) [6–8]. CFTR is a cyclic adenosine monophosphate (cAMP)-regulated Cl^- channel located at the apical membrane of epithelial cells in several tissues. CFTR expression is developmentally regulated and shows temporal and tissue-specific distribution during fetal lung growth with its highest expression in the first and second trimester of gestation and a gradual decline in the third trimester [9–11]. In the postnatal lung, CFTR expression is largely limited to cells of the submucosal glands and individual cells of the airway [12,13]. By contrast, a large majority of cells express CFTR during fetal lung development [9] and its expression is about 75-fold higher than in the adult lung [14]. Timings of high and low CFTR expression correspond to the developmental stages of the lung. In sheep, high Cftr expression was observed during the pseudoglandular stage whereas Cftr expression decreases during the late canalicular stage [14]. This expression pattern suggests a role of CFTR in fetal lung development which has yet to be determined. Evidence for this assumption comes from animal studies analyzing transient knock-out and over-expression of Cftr during fetal development. Cftr over-expression in the pseudoglandular stage accelerated epithelial cell proliferation resulting in an increased lung surface area, saccular density and an increased number of air spaces [7]. By contrast, a transient *in utero* Cftr knock-out led to a cystic fibrosis (CF)-like phenotype [8,15]. These studies showed that even small or transient alteration of Cftr expression in the developing lung had profound consequences for lung organogenesis.

It is known that fluid absorption through epithelial Na^+ transport is under hormonal control since the epithelial Na^+ channel (ENaC) is regulated by different hormones, like cortisol, aldosterone and epinephrine [16–18]. Little is known about the effect of hormones on CFTR. However, it is noteworthy that the fetal cortisol level rises in the same developmental stage of lung development in which CFTR expression declines. Therefore, the goal of our study was to determine the effect of glucocorticoids (GCs) on CFTR mRNA expression and channel activity in different pulmonary cell types, analyze the mode of action and thereby provide a possible link between the fetal CFTR expression pattern and the fetal cortisol levels.

Materials and Methods

Tissue preparation

All animal care and experimental procedures were approved by the institutional review board (Landesdirektion Leipzig, Permit Number: T36/13). Sprague-Dawley rats were bred at the Medical Experimental Center (MEZ) of Leipzig University. Animals were housed in rooms

with a controlled temperature (22°C), humidity (55%) and 12 h light-dark cycle. Food and water were freely available. Rats were euthanized by carbon dioxide inhalation.

Fetal distal lung epithelial (FDLE) cell isolation and culture. FDLE cells, a model of respiratory cells in the late canalicular / early saccular stage of lung development, were isolated from lungs of 19–20 d gestation fetal rats as described previously [19,20]. In brief, lungs were minced and digested in a solution with 0.125% trypsin (Life Technologies, Darmstadt, Germany) and 0.4 mg/ml DNAse (# LS006333, CellSystems, Troisdorf, Germany) in Hanks' Balanced Salt Solution (HBSS) (Life Technologies) for 10 min at 37°C. Digestion was stopped by adding Minimum Essential Eagle's Medium (MEM) (Life Technologies) containing 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany). Cells were collected, centrifuged (440 x g) and re-suspended in 15 ml MEM containing 0.1% collagenase (# LS004194, CellSystems) and DNAse for further digestion. The solution was incubated for 15 min at 37°C. Collagenase activity was stopped by adding 15 ml MEM containing 10% FBS. Cells were plated twice for 1 h to remove contaminating fibroblasts. The supernatant contained epithelial cells with > 95% purity [20]. For Ussing chamber measurements, cells were seeded on permeable Snapwell supports (Costar # 3407, Corning, Inc., NY, surface area 1 cm^2) at a density of 10^6 cells per insert. For real-time quantitative PCR (RT-qPCR) analyses, cells were seeded on permeable Transwell supports (Costar # 3412, surface area 4.5 cm^2) at a density of $2x10^6$ cells per insert. FDLE cells were cultured under submerged conditions and medium was changed daily. The culture medium contained MEM with 10% FBS, 2 mM L-glutamine (Life Technologies), penicillin (100 U/ml, Life Technologies), streptomycin (100 μg/ml, Life Technologies) and amphotericin B (0.25 μg/ml, Life Technologies). For all analyzed cell types serum-free complete medium (Cellgro, Mediatech, Herndon, VA), supplemented with dexamethasone (Sigma-Aldrich, Taufkirchen, Germany) was added 24 h before measurement or as stated otherwise. Measurements of FDLE monolayers were performed 72–96 h after isolation. At this time point monolayers exhibited a high transepithelial resistance (R_{te}) of 997.3 ± 424.2 Ω^*cm^2 (mean ± SD). Longer incubation times are not recommended since alveolar type II (ATII) cells have been described to transition into alveolar type I (ATI) cells after prolonged incubation *in vitro* [21]. To determine the involvement of the glucocorticoid receptor (GR), mifepristone (10 μM, # M-8046, Sigma-Aldrich) was used. Cells subjected to different experimental conditions were always age matched, derived from the same litter, treated equally and recorded simultaneously.

Isolation of adult ATII cells. A complete description can be found elsewhere [22]. In brief, Sprague-Dawley rats (140–200 g) were anesthetized (ketamin 10% and xylazil 2%), and injected with heparin (400 IU/kg). The lungs were perfused with saline solution and removed from the body. Resected lungs were washed repeatedly and incubated twice with a saline solution containing 0.25 mg/ml elastase (# EC134, Elastin Products Company Inc., MO, USA) and 0.05 mg/ml trypsin at 37°C for 15 min. Afterwards lung tissue was dissected from the bronchi and trachea and sliced into 1 mm bits within a DNase containing solution. Enzyme reaction was stopped by incubation with FBS (37°C, 2 min). The tissue was filtered 3 times through gauze and nylon meshes (mesh width: 120, 20, and 10 μm) and the filtrate centrifuged for 8 min at 130 x g. After re-suspending in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies), cells were incubated on IgG (Life Technologies) coated plastic dishes at 37°C for 15 min. Non-adhering cells were again centrifuged for 8 min at 130 x g and re-suspended in DMEM with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded according to the FDLE cells, and measurements and RNA isolation performed 96 h after isolation. Their mean R_{te} was 814.1 ± 422.3 Ω^*cm^2.

Isolation of primary airway epithelial cells. Isolation of primary airway epithelial cells has been described previously [23]. Briefly, the trachea proximal to the bronchial bifurcation was isolated from euthanized male Sprague-Dawley rats (140–200 g). Esophageal remnants

and adherent adipose tissue were removed. The trachea was opened longitudinally and rinsed with DMEM before incubation in DMEM with 0.1% protease XIV (# P5147, Sigma-Aldrich), 0.01% DNase and 1% FBS for 21 h at 4°C. The digestion was stopped by the addition of 1 Vol. FBS. The trachea was then agitated and scraped with a cell scraper to detach the airway epithelial cells. The obtained cell suspension was centrifuged twice at 500 x g for 5 min and re-suspended in cell culture media. Cell culture media consisted of DMEM-F12 (Life Technologies) with 1 μg/ml insulin (# 91077C, Sigma-Aldrich), 7.5 μg/ml transferrin (# 354204, Corning), 1 μM hydrocortisone (# H0888, Sigma-Aldich), 30 nM 3,5,3'-triiodothyronine (# T6397, Sigma-Aldrich), 2.5 ng/ml epidermal growth factor (# 354052, Corning), 10 ng/ml endothelial cell growth supplement (# 354006, Corning), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) which was supplemented (1:1) with 3T3 fibroblast (from ATCC, # CCL-92)-conditioned DMEM containing 2% FBS. Cells were seeded on collagen-coated permeable supports at a density of $2x10^5$ cells per Snapwell insert and $4x10^5$ cells per Transwell insert. Measurements and RNA isolation were done approximately 10 days after plating, when R_{te} reached values >300 Ω^*cm^2 (mean R_{te}: 2568 ± 2097 Ω^*cm^2).

Culture of cell lines

A549 cells (from ATCC, # CCL-185), an adenocarcinoma-derived human alveolar epithelial cell line, and Calu-3 cells (from ATCC, # HTB-55), derived from human bronchial submucosal glands, were kindly provided by Dr. Getu Abraham (Institute of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, University of Leipzig). A549 cells (passage 10–26) were cultured in DMEM with 10% FBS and Calu-3 cells (passage 23–30) in DMEM-F12 with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% non-essential amino acids (Life Technologies), and passaged 1–2 times weekly. A549 cells were seeded on Transwell supports at a density of $2x10^6$ cells per insert according to the FDLE cells and RNA was isolated after 5 days. Calu-3 cells were seeded at a density of $5x10^5$ per Snapwell insert and $1x10^6$ per Transwell insert. After 10 days, Calu-3 cells were subjected to air-liquid interface conditions and R_{te} was measured every two days during medium change with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) with STX-2 chopstick electrodes. Measurements and RNA isolation were done approximately 14–21 days after plating of Calu-3 cells on permeable supports, when R_{te} reached values >300 Ω^*cm^2 (mean R_{te}: 888.2 ± 400.8 Ω^*cm^2). The 16HBE14o- cell line (passage 2.82–86) was generated from human bronchial surface epithelium as described previously [24] and was kindly provided by Dr. Dieter Gruenert (Mt Zion Research Center, University of California, San Francisco, CA, USA). The 16HBE14o- cells were grown in MEM with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell culture flasks and permeable supports were coated with a solution containing bovine collagen I (Corning), human fibronectin (Corning) and bovine serum albumin (Sigma-Aldrich) in LHC basal medium (Life Technologies). Cells were seeded at a density of $5x10^5$ per Transwell insert. After 7 days, 16HBE14o$^-$ cells were subjected to air-liquid interface conditions and after 14 days RNA isolation was performed.

To determine the involvement of the phosphoinositide 3-kinase (PI3K), the inhibitor LY-294002 (10 μM, # 1130 TOCRIS Bioscience, Bristol, UK) was used.

Measurement of mRNA expression

Total RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) and treated with DNase I (Life Technologies) according to the manufacturer's instructions. Reverse transcription was carried out in two steps by first pre-annealing of 1 μg RNA with $Oligo(dT)_{18}$ primers (ThermoFisher Scientific, St. Leon-Rot, Germany), followed by the addition of Superscript III

Table 1. Gene-specific primers.

gene	forward	reverse
CFTR-humanNM_000492.3	GGGCTGTGTCCTAAGCCATGGCCA	GATGGCTTGCCGGAAGAGGCTCC
Cftr-ratNM_031506.1	GCCTTCGCTGGTTGCACAGTAGTC	GCTTCTCCAGCACCCAGCACTAGA
MRPS18A-humanNM_001193343.1	CGGCTTCCAGCTCGCGGGTTC	GGTACCTGCTCGGTGGGCCATC
Mrps18a-ratNM_198756.1	GCGACCGGCTGGTTATGGCT	GGGCACTGGCCTGAGGGATTAG
ANO1-humanNM_018043.5	CGGAAACAGATGCGACTCAAC	TGATCCTTGACAGCCTCCTCTT
Ano1-ratNM_001107564.1	CCTGTTCGTTGCGTCCTTCCCTC	GAGCGTGTGGTTGACGAAGCCG
ANO6-human (a-d) NM_001025356.2NM_001142678.1NM_001142679.1NM_001204803.1	CAGCCACCAGAAGCCGCATTG	CTCTGACTGACGGCGGAATTTGC
Ano6-ratNM_001108108.1	GGGACCCGGTGTACTGGCTGG	CATAGAACAATCCCAGCCTGCCC
CLC5-V2/5-humanNM_001127898.3NM_001272102.1	GCCCCGAGTTTGGGGCTTTA	CCCTGCTGAAAGCCTCTGTTATCC
Clc5-ratNM_017106.1	CGTGGCTTGCTGCTGTGGGAA	GAGAGCAGCGAAGAAGGAACGCC
NKCC1-humanNM_001256461.1	GAATCCAAAGGCCCTATTGTGCC	GCCATCGCTCTCCGGTCATG
Nkcc1-ratNM_031798.1	GGCCATCGCTGACTTCGTCATAGG	GCAAGGTCACCCGAGATGTTCGC

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(Life Technologies) and incubation at 55°C for 1 h and 75°C for 15 min. The resulting cDNA was diluted 1:10 in Tris-EDTA buffer (AppliChem, Darmstadt, Germany) and the Platinum Taq polymerase (Life Technologies) was used for RT-qPCR, following the manufacturer's instructions. Reactions were conducted with the CFX 96 Real-Time system (BioRad, Munich, Germany) with SYBR-Green (Molecular Probes, Eugene Oregon, USA). Transcripts of target genes were amplified using the gene-specific primers listed in Table 1. Absolute quantification was performed using a several fold dilution of target specific plasmid DNA as internal standard curve. Amplification efficiencies were close to 100%, calculated by standard curve analysis and standard curve plots showed a high coefficient of determination (R^2>0.99). The resulting molecule concentrations were normalized to a reference gene (Mrps18a: mitochondrial ribosomal protein S18a). Constant expression of Mrps18a was confirmed against other common reference genes. The fold change of mRNA levels was calculated with the relative standard curve method. Measurements were performed in technical triplicates and at least 3 biological replicates. Melting curves and gel electrophoresis of PCR products were routinely performed to determine the specificity of the PCR reaction.

Electrophysiological measurements

A detailed description of Ussing chamber measurement procedures is reported elsewhere [19]. Experiments were included in the data analyses only when R_{te} exceeded 300 $\Omega^* cm^2$ throughout the measurement. Ussing chambers were filled with a ringer solution containing: Na^+ 145 mM, K^+ 5 mM, Ca^{2+} 1.2 mM, Mg^{2+} 1.2 mM, Cl^- 125 mM, HCO_3^- 25 mM, $H_2PO_4^-$ 3.3 mM, HPO_4^{2-} 0.8 mM (pH 7.4). The basolateral side contained 10 mM glucose whereas 10 mM mannitol was used in the apical compartment. Equivalent short-circuit currents (I_{SC}) were assessed every 20 s by measuring transepithelial voltage (V_{te}) and R_{te} using a transepithelial current clamp (Physiologic Instruments, San Diego, CA), and calculating the quotient $I_{SC} = V_{te}/R_{te}$. Amiloride (10 μM, # A-7410, Sigma-Aldrich), an inhibitor of ENaC, was added to the apical compartment to inhibit amiloride-sensitive Na^+ channels. Forskolin (10 μM, # F-6886, Sigma-Aldrich) was added to the apical compartment to increase the intracellular cAMP concentration and thereby activate cAMP-sensitive ion channels like CFTR. Finally, glibenclamide (200 μM, # G-0639, Sigma-Aldrich) or $CFTR_{inh}172$ (10 μM, # 3430, TOCRIS Bioscience) was applied apically to determine the glibenclamide-sensitive or $CFTR_{inh}172$-sensitive I_{SC}, a measure of CFTR activity. In another experiment measurements were carried out in Cl^--free solution containing: Na^+ 145

mM, K^+ 5 mM, Ca^{2+} 4 mM, Mg^{2+} 1 mM, gluconate 125 mM, HCO_3^- 25 mM, $H_2PO_4^-$ 3.3 mM, HPO_4^{2-} 0.8 mM (pH 7.4) or HCO_3^--free solution containing: Na^+ 145 mM, K^+ 5 mM, Ca^{2+} 1.2 mM, Mg^{2+} 1.2 mM, Cl^- 145 mM, $H_2PO_4^-$ 3.3 mM, HPO_4^{2-} 0.8 mM (pH 7.4). In HCO_3^--free measurements Ussing chambers were gassed with 100% O_2 instead of 95% O_2 / 5% CO_2.

Amiloride was dissolved in water; forskolin, glibenclamide, $CFTR_{inh}172$, mifepristone and LY-294002 were prepared in DMSO and dexamethasone in 100% ethanol. Control monolayers were treated with the respective solvent to exclude solvent influence on the evoked responses.

Statistical analysis

For statistical analyses, GraphPad Prism (version 5.03; GraphPad Software, Inc, San Diego, CA, USA) was used. Differences among groups treated with dexamethasone and controls were evaluated by unpaired T-test, or analysis of variance (ANOVA) followed by Dunnett's or Tukey's *post hoc* test, as appropriate. A probability of $p<0.05$ was considered significant for all statistical analyses.

Results

Effect of dexamethasone on CFTR/Cftr expression and activity in alveolar cells

Cftr mRNA expression was determined in FDLE cells incubated in medium containing different dexamethasone concentrations (1 nM, 10 nM, 50 nM, 100 nM, 300 nM and 1 μM for 24 h) and compared to control monolayers without hormone addition. Cftr mRNA expression decreased with increasing dexamethasone concentrations in a dose-dependent manner, starting at 10 nM dexamethasone (Fig 1A). At 100 nM dexamethasone, Cftr mRNA expression was maximally inhibited, with mRNA expression levels decreased by 70% ($p<0.001$). The time course of the dexamethasone modulation was determined at different time points (6, 12 and 24 h) using 100 nM dexamethasone demonstrating a reduction of Cftr mRNA expression within 6 h ($p<0.05$) that further decreased thereafter (Fig 1B).

Next, we determined the effect of 100 nM dexamethasone on Cftr channel activity in Ussing chambers. Following FDLE cell isolation, cell culture media was changed to serum-free medium after 24 h and monolayer were divided into four groups. Control monolayers were cultured in serum-free medium for 72 h, whereas the other groups were supplemented with

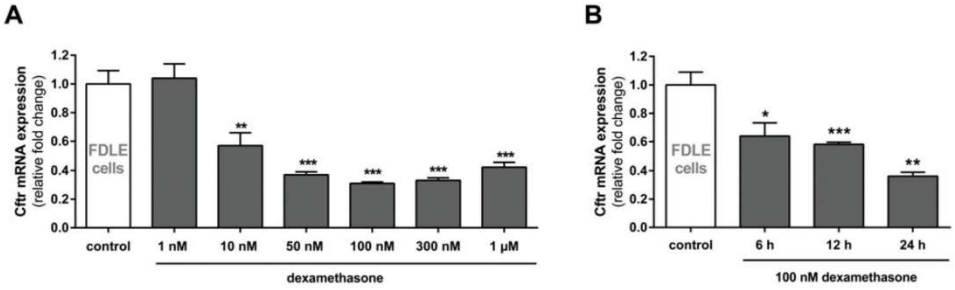

Fig 1. Dexamethasone reduces Cftr mRNA expression in FDLE cells. Graphs represent the mean + SEM for normalized Cftr mRNA expression acquired by RT-qPCR. **A:** Dose-response curve of dexamethasone effect (1 nM–1 μM for 24 h, n = 4, ** p<0.01; *** p<0.001, ANOVA with Dunnett's *post hoc* test compared to control monolayers without dexamethasone addition). **B:** Time course of Cftr mRNA expression in response to 100 nM dexamethasone for 6, 12 and 24 h (n = 4, * p<0.05; ** p<0.01; *** p<0.001, T-test).

doi:10.1371/journal.pone.0124833.g001

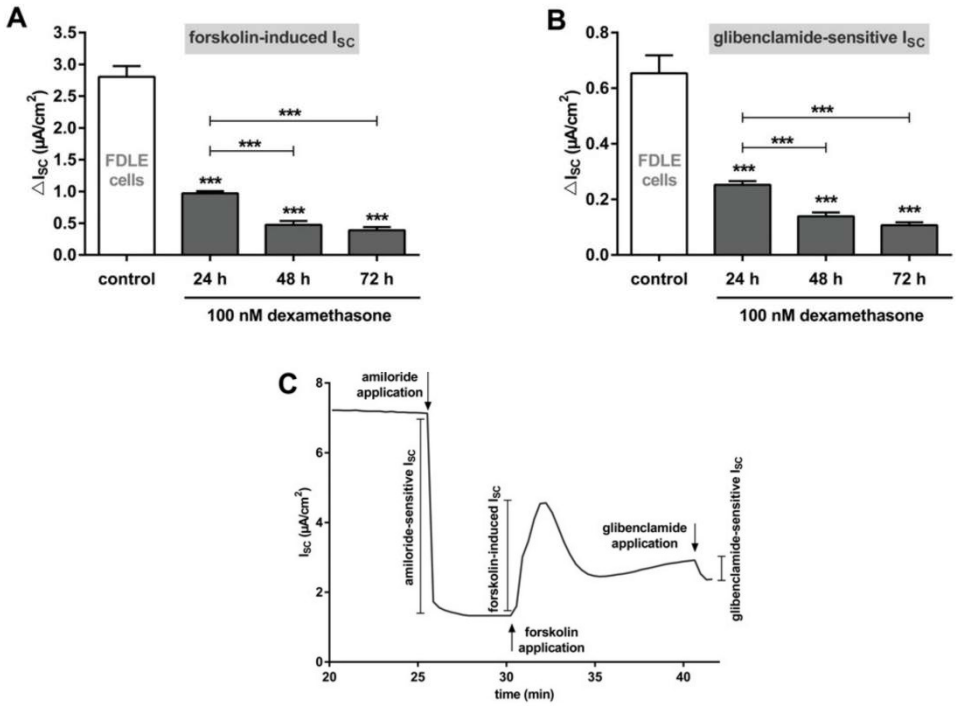

Fig 2. Dexamethasone reduces Cftr channel activity in FDLE cells. Graphs represent the mean + SEM of I_{SC} in response to 100 nM dexamethasone for 24, 48 and 72 h measured in Ussing chambers. **A:** Forskolin-induced I_{SC} (n = 25–56, *** p<0.001, ANOVA with Tukey's *post hoc* test). **B:** Glibenclamide-sensitive I_{SC} (n = 25–55, *** p<0.001, ANOVA with Tukey's *post hoc* test). **C:** Typical current tracing of FDLE monolayers.

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dexamethasone for either, 24 h, 48 h or 72 h. Ussing chamber measurements were performed 96 h after cell isolation. After the basal current reached a plateau, amiloride was applied to inhibit amiloride-sensitive Na^+ channels like ENaC, followed by the addition of forskolin to maximally stimulate existing Cftr channels alone without interference from ENaC. Finally, glibenclamide was added to inhibit Cftr activity (Fig 2C). After calculating the forskolin-induced and glibenclamide-sensitive I_{SC}, a measure of CFTR activity, dexamethasone-stimulated FDLE monolayers were compared to unstimulated control monolayers. FDLE monolayers treated with dexamethasone for 24 h showed a decreased Cftr activity by more than 65%, as shown by the significantly reduced forskolin-induced and glibenclamide-sensitive I_{SC} ($p<0.001$, Fig 2A and 2B). Prolonged dexamethasone treatment for 48 and 72 h further decreased the forskolin-induced and glibenclamide-sensitive I_{SC} ($p<0.001$, Fig 2A and 2B).

Since FDLE cells represent a fetal primary cell culture, primary adult ATII cells were analyzed to examine if the observed effects were dependent on the developmental stage of the cells. 100 nM dexamethasone also significantly reduced Cftr mRNA expression in adult ATII cells ($p<0.001$, Fig 3A). Furthermore, Cftr channel activity measured as forskolin-induced I_{SC} in monolayers treated with 100 nM dexamethasone for 24 h was significantly reduced ($p<0.05$,

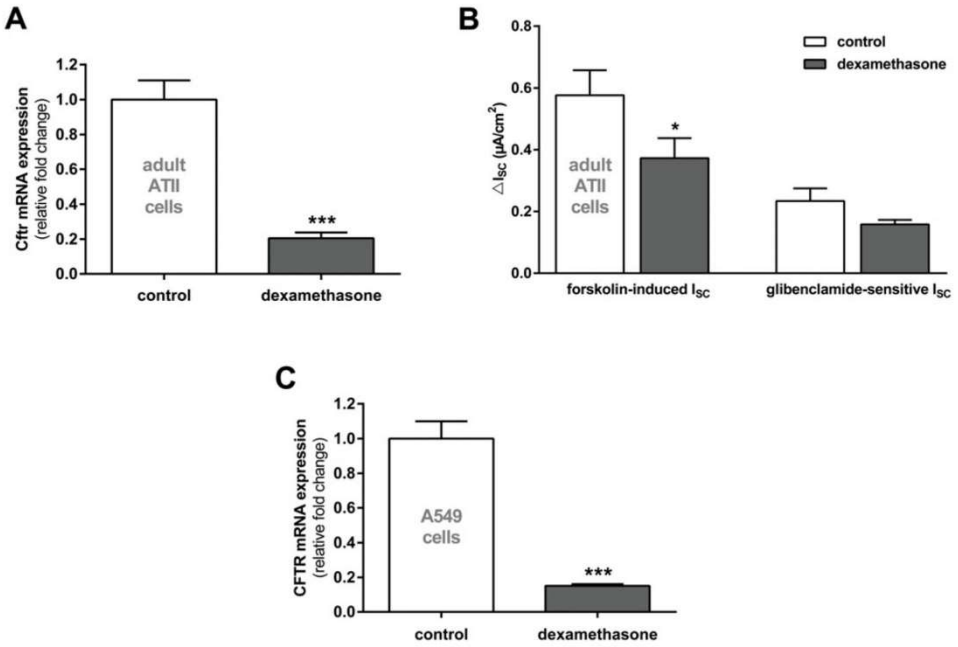

Fig 3. Dexamethasone reduces CFTR/Cftr mRNA expression and channel activity in adult alveolar cells. A/B: primary adult ATII cells. **A:** Graph represents the mean + SEM for normalized Cftr mRNA expression in response to 100 nM dexamethasone for 24 h acquired by RT-qPCR (n = 6, *** p<0.001 by T-test compared to control monolayers without dexamethasone addition). **B:** Graph represents the mean + SEM of I_{SC} in response to 100 nM dexamethasone for 24 h measured in Ussing chambers. Forskolin-induced I_{SC} (n = 29–31, * p<0.05 by T-test compared to control monolayers without dexamethasone addition) and glibenclamide-sensitive I_{SC} (n = 27–28). **C:** A549 cells. Graph represents the mean + SEM for normalized CFTR mRNA expression in response to 100 nM dexamethasone for 24 h acquired by RT-qPCR (n = 8, *** p<0.001, T-test compared to control cells without dexamethasone addition).

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Fig 3B), and the glibenclamide-sensitive I_{SC} demonstrated a non-significant trend in the same direction. To determine whether the analyzed effect of dexamethasone on Cftr in alveolar cells was species-specific the analysis of CFTR mRNA expression was repeated with the human alveolar cell line A549. Results showed that 100 nM dexamethasone for 24 h also reduced CFTR mRNA expression in human A549 cells by 80% ($p<0.001$, Fig 3C).

To determine the pathway of dexamethasone action we used mifepristone to block the GR. Inhibition by mifepristone restored Cftr mRNA expression and channel activity in FDLE cells (Fig 4). Cftr mRNA expression analysis showed that mifepristone by itself reduced Cftr expression ($p<0.05$, Fig 4A) yet addition of dexamethasone did not further decrease Cftr expression in the presence of mifepristone. By contrast, Cftr expression in the sole presence of dexamethasone was significantly lower than in mifepristone- or mifepristone/dexamethasone-incubated FDLE cells ($p<0.001$). Cftr activity in dexamethasone-incubated FDLE monolayers was restored to control levels by mifepristone and mifepristone by itself did not affect Cftr activity as shown for the forskolin-induced and $CFTR_{172}$inh-sensitive I_{SC} in Ussing chambers ($p<0.001$, Fig 4B and 4C).

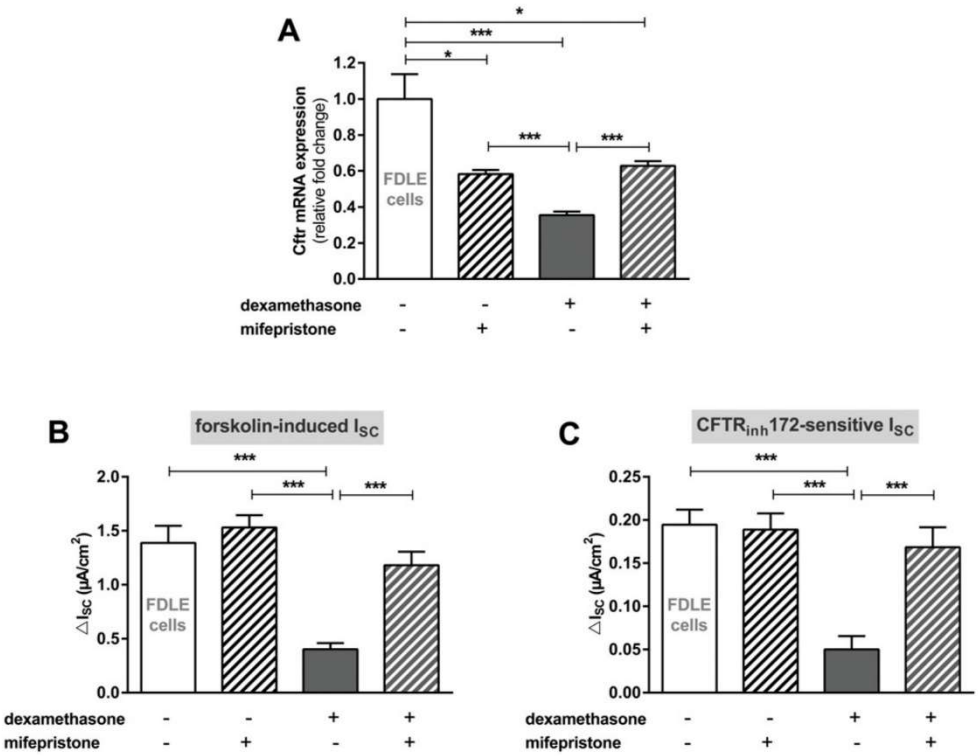

Fig 4. Mifepristone restores Cftr mRNA expression and channel activity in the presence of dexamethasone in FDLE cells. A: Graph represents the mean + SEM for normalized Cftr mRNA expression in response to 100 nM dexamethasone and mifepristone for 24 h acquired by RT-qPCR (n = 4, * p<0.05, *** p<0.001, ANOVA with Tukey's *post hoc* test). **B/C:** Graphs represent the mean + SEM of I_{SC} in response to 100 nM dexamethasone and mifepristone for 24 h measured in Ussing chambers. **B:** Forskolin-induced I_{SC} (n = 10–17, *** p<0.001, ANOVA with Tukey's *post hoc* test). **C:** $CFTR_{inh}172$-sensitive I_{SC} (n = 8–13, *** p<0.001, ANOVA with Tukey's *post hoc* test).

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Effect of dexamethasone on CFTR/Cftr expression and activity in airway-derived cells

Next, we sought to determine whether the effect of dexamethasone on CFTR was related to the cell type. Therefore, we analyzed the effect of 100 nM dexamethasone for 24 h in human bronchial submucosal gland-derived Calu-3 cells. Similar to alveolar cells, treatment of Calu-3 cells with dexamethasone led to a significant reduction of CFTR mRNA expression ($p<0.001$, Fig 5A). However, the extent by which the CFTR expression level was reduced differed between alveolar and bronchial cells. In Calu-3 cells mRNA expression was reduced by approximately 20% compared to control cells whereas in alveolar cells CFTR/Cftr expression diminished by 70–80%. By contrast, CFTR channel activity, as measured in Ussing chambers, was actually increased by 100 nM dexamethasone. As shown in Fig 5B and 5C, treatment of Calu-3 cells with dexamethasone led to a significantly increased forskolin-induced and glibenclamide-sensitive I_{SC} ($p<0.01$, $p<0.001$). Since CFTR is permeable for both Cl^- or HCO_3^- ions, we repeated the

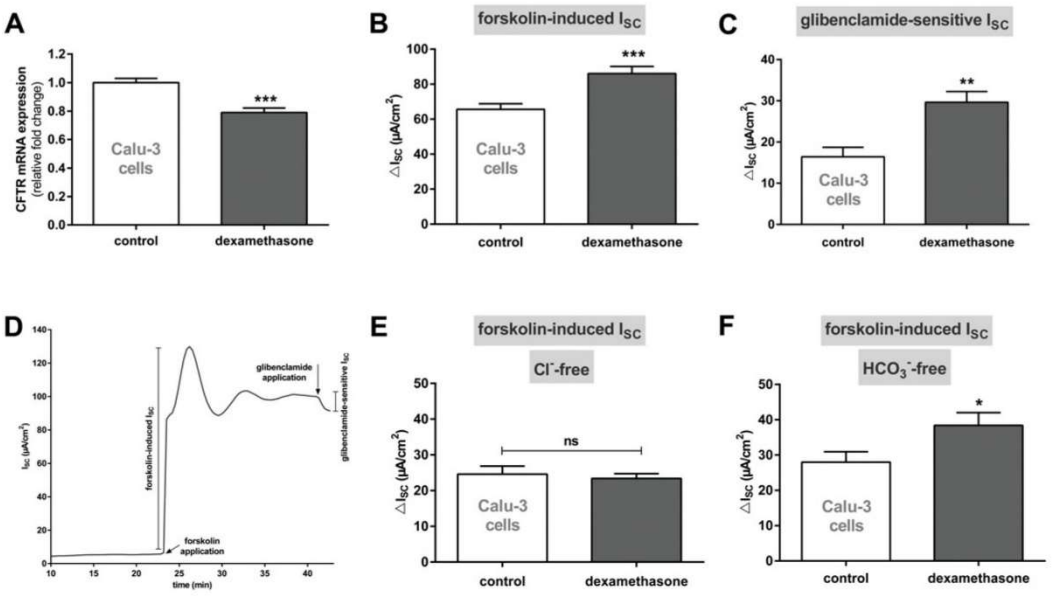

Fig 5. Dexamethasone reduces CFTR mRNA expression and increases channel activity in Calu-3 cells. A: Graph represents the mean + SEM for normalized CFTR mRNA expression in response to 100 nM dexamethasone for 24 h acquired by RT-qPCR (n = 6, *** p<0.001, T-test compared to control cells without dexamethasone addition). **B/C/E/F:** Graphs represent the mean + SEM of I_{SC} in response to 100 nM dexamethasone for 24 h measured in Ussing chambers. **B:** Forskolin-induced I_{SC} (n = 12, *** p<0.001, T-test compared to control cells without dexamethasone addition). **C:** Glibenclamide-sensitive I_{SC} (n = 6, ** p<0.01, T-test compared to control cells without dexamethasone addition). **D:** Typical current tracing of Calu-3 cells. **E:** Forskolin-induced I_{SC} measured in Cl^--free solution (n = 14–16, ns = not significant). **F:** Forskolin-induced I_{SC} measured in HCO_3^--free solution (n = 16, * p<0.05, T-test compared to control cells without dexamethasone addition).

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measurements in solutions either without Cl^- or HCO_3^-. Omitting Cl^- abolished the effect of dexamethasone on the forskolin-induced I_{SC} (Fig 5E). By contrast, omitting HCO_3^- did not affect the stimulation of forskolin-induced I_{SC} by dexamethasone which was significantly higher compared to control cells ($p<0.05$, Fig 5F).

Furthermore, experiments were repeated with human bronchial 16HBE14o- cells incubated with 100 nM dexamethasone for 24 h. In accordance, CFTR mRNA expression was reduced by 50% in the presence of dexamethasone ($p<0.001$, Fig 6A). Since Calu-3 and 16HBE14o- cells represent immortalized cell lines we further analyzed the effect of dexamethasone on Cftr expression and activity in primary rat airway epithelial cells. Likewise, Cftr mRNA expression was reduced by 100 nM dexamethasone for 24 h in primary airway epithelial cells ($p<0.001$, Fig 6B). CFTR activity was increased as demonstrated by the significantly elevated forskolin-induced and $CFTR_{172}$inh-sensitive I_{SC} in primary airway epithelial cells ($p<0.01$, Fig 6D and 6E). Therefore, results obtained with Calu-3 and 16HBE14o- cells were confirmed by primary airway epithelial cells.

Measurements using Calu-3 cells were also performed in the presence of mifepristone to address the function of the GR in these cells. Mifepristone restored CFTR mRNA expression because no difference was observed between mifepristone- and mifepristone/dexamethasone-incubated Calu-3 cells (Fig 7A). In addition, the dexamethasone-induced increase of forskolin-

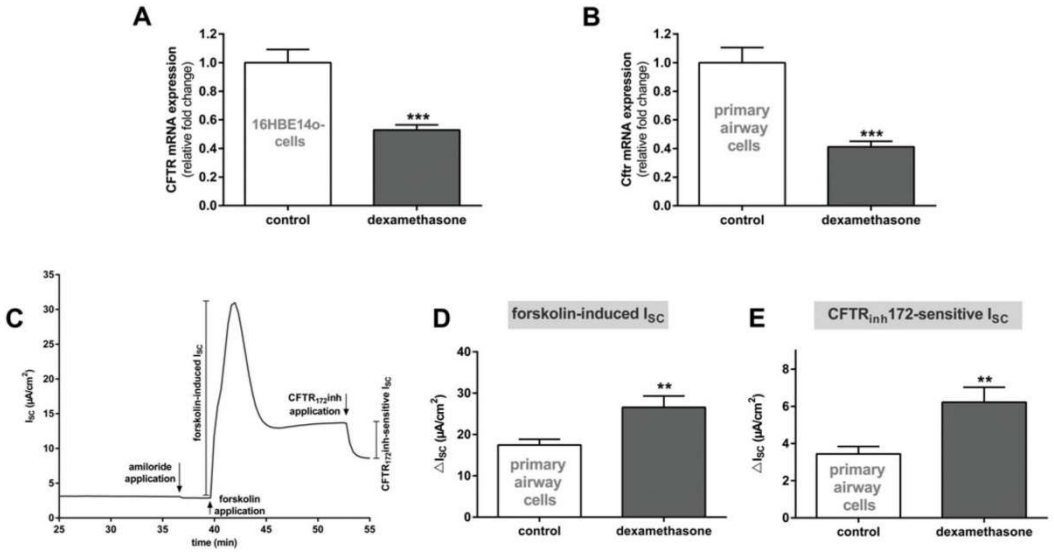

Fig 6. Dexamethasone reduces CFTR/Cftr mRNA expression and increases channel activity in airway epithelial cells. A/B: Graphs represent the mean + SEM for normalized CFTR/Cftr mRNA expression in response to 100 nM dexamethasone for 24 h acquired by RT-qPCR. **A:** 16HBE14o- cells (n = 8, *** p<0.001, T-test compared to control cells without dexamethasone addition). **B:** Primary rat airway epithelial cells (n = 7–8, *** p<0.001, T-test compared to control cells without dexamethasone addition). **C:** Typical current tracing of primary airway epithelial cells. D/E: Graphs represent the mean + SEM of I_{SC} in response to 100 nM dexamethasone for 24 h measured in Ussing chambers. **D:** Forskolin-induced I_{SC} (n = 20–23, ** p<0.01, T-test compared to control cells without dexamethasone addition). E: $CFTR_{172}$inh-sensitive I_{SC} (n = 19–20, ** p<0.01, T-test compared to control cells without dexamethasone addition).

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induced and $CFTR_{172}$inh-sensitive I_{SC} measured in Ussing chambers was indistinguishable from measurements without dexamethasone when mifepristone was present (Fig 7B and 7C).

Since dexamethasone reduces CFTR mRNA expression in Calu-3 cells, the increase of CFTR activity has to be achieved by post-translational mechanisms. Because kinases are known to influence the activity of ion channels, we determined whether the PI3K is involved. Inhibiting the PI3K with LY-294002 decreased CFTR activity in control and dexamethasone-stimulated cells ($p<0.05$, $p<0.01$, $p<0.001$, Fig 8A and 8B). Furthermore, dexamethasone was unable to increase CFTR activity when LY-294002 was present.

Effect of dexamethasone on mRNA expression of alternative chloride transporters

Finally, we determined the effect of dexamethasone on alternative Cl^- transporters in alveolar (Fig 9) and bronchial cells (Fig 10). Anoctamine 1 [ANO1/Ano1, TMEM16A] mRNA expression was decreased by dexamethasone in all analyzed alveolar cell types ($p<0.01$, $p<0.001$, Fig 9A–9C) and increased in bronchial Calu-3 and 16HBE14o- cells ($p<0.05$, $p<0.01$, Fig 10A and 10B). Furthermore, anoctamin 6 [ANO6/Ano6, TMEM16F] and voltage-gated chloride channel 5 [CLC5/Clc5] mRNA expression were increased by dexamethasone in alveolar cells ($p<0.05$, $p<0.001$, Fig 9A–9C) yet no effect was seen in Calu-3 and 16HBE14o- cells (Fig 10A and 10B). NKCC1/Nkcc1 mRNA expression was decreased by dexamethasone in alveolar and also in bronchial cells alike ($p<0.01$, $p<0.001$, Figs 9A–9C; 10A and 10B).

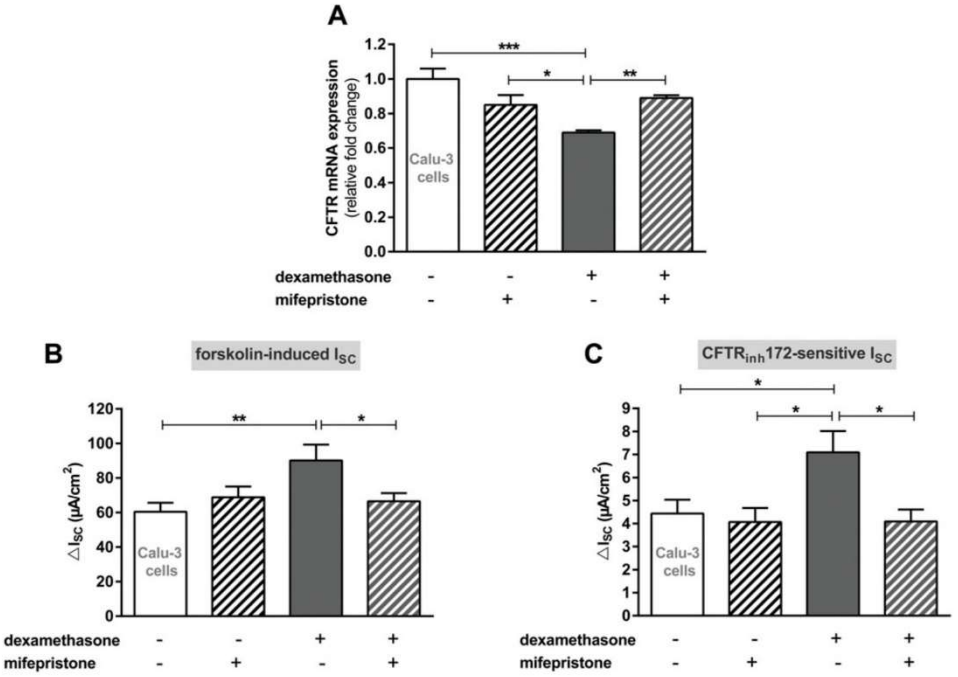

Fig 7. Mifepristone restores CFTR mRNA expression and reduces channel activity in the presence of dexamethasone in Calu-3 cells. A: Graph represents the mean + SEM for normalized CFTR mRNA expression in response to 100 nM dexamethasone and mifepristone for 24 h acquired by RT-qPCR (n = 3–4, * p<0.05, ** p<0.01, *** p<0.001, ANOVA with Tukey's *post hoc* test). **B/C:** Graphs represent the mean + SEM of I_{SC} in response to 100 nM dexamethasone and mifepristone for 24 h measured in Ussing chambers. **B:** Forskolin-induced I_{SC} (n = 7–12, * p<0.05, ** p<0.01, ANOVA with Tukey's *post hoc* test). **C:** $CFTR_{inh}172$-sensitive I_{SC} (n = 7–12, * p<0.05, ANOVA with Tukey's *post hoc* test).

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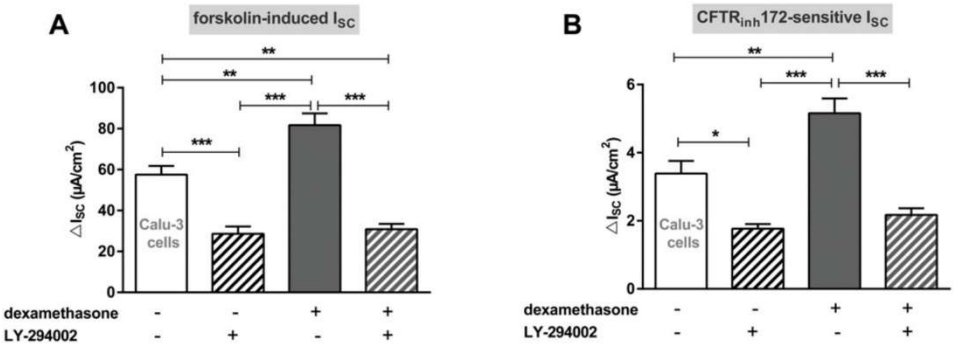

Fig 8. LY-294002 prevents the increase of CFTR activity induced by dexamethasone in Calu-3 cells. Graphs represent the mean + SEM of I_{SC} in response to 100 nM dexamethasone and LY-294002 for 24 h measured in Ussing chambers. **A:** Forskolin-induced I_{SC} (n = 12–18, ** p<0.01, *** p<0.001, ANOVA with Tukey's *post hoc* test). **B:** $CFTR_{inh}172$-sensitive I_{SC} (n = 12–18, * p<0.05, ** p<0.01, *** p<0.001, ANOVA with Tukey's *post hoc* test).

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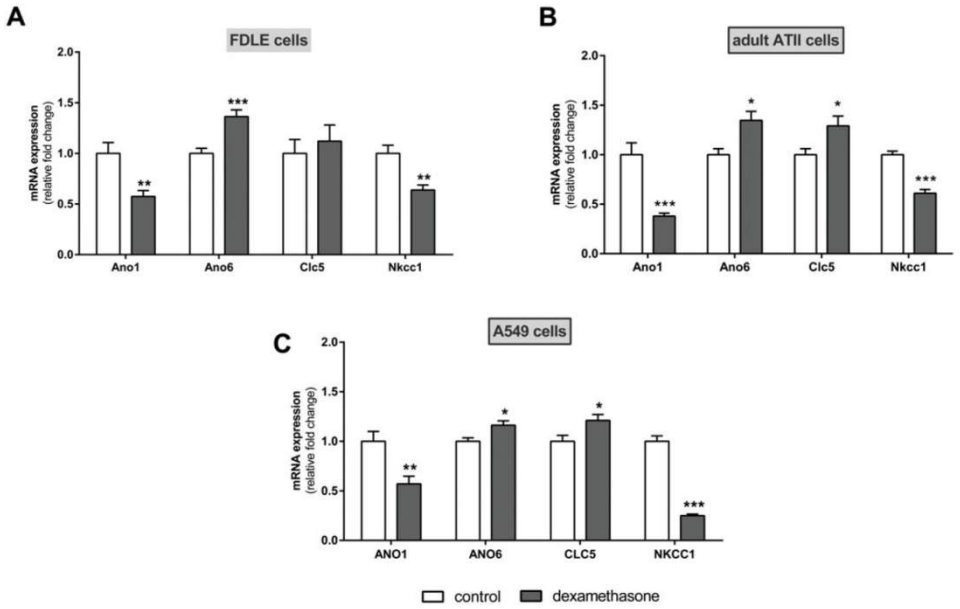

Fig 9. Dexamethasone modulates alternative chloride transporters in alveolar cells. Graphs represent the mean + SEM for normalized mRNA expression in response to 100 nM dexamethasone for 24 h acquired by RT-qPCR. **A:** FDLE cell monolayers (n = 7–12, ** p<0.01, *** p<0.001, T-test compared to control monolayers without dexamethasone addition). **B:** Adult ATII cells (n = 4–6, * p<0.05, *** p<0.001, T-test compared to control monolayers without dexamethasone addition). **C:** A549 cells (n = 8–12, * p<0.05, ** p<0.01, *** p<0.001, T-test compared to control cells without dexamethasone addition).

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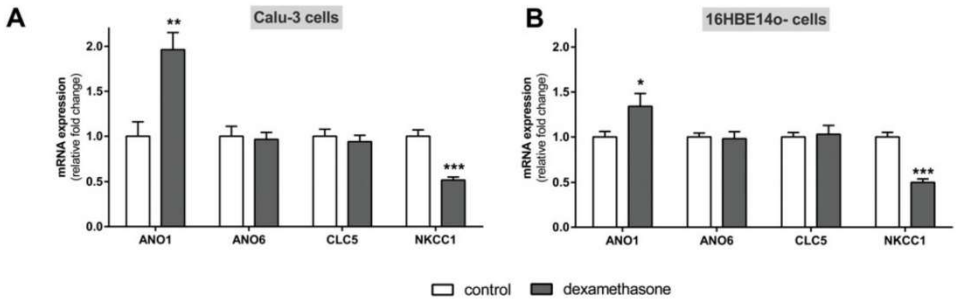

Fig 10. Dexamethasone modulates alternative chloride transporters in bronchial cells. Graphs represent the mean + SEM for normalized mRNA expression in response to 100 nM dexamethasone for 24 h acquired by RT-qPCR. **A:** Calu-3 cells (n = 8–14, ** p<0.01, *** p<0.001, T-test compared to control cells without dexamethasone addition). **B:** 16HBE14o- cells (n = 7–8, * p<0.05, *** p<0.001, T-test compared to control cells without dexamethasone addition).

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Discussion

The study shows that dexamethasone reduces CFTR/Cftr mRNA expression in a concentration dependent manner. The strongest inhibition was achieved with 100 nM dexamethasone, which reduced the Cftr expression level by 70%. Dexamethasone concentrations above 100 nM did not further decrease Cftr expression. Noteworthy, a lower effect of higher dexamethasone concentrations on ENaC mRNA expression has been described before [25]. The response to GC treatment occurred within hours of exposure and a down-regulation of Cftr expression was detected only 6 h after dexamethasone addition. Cftr expression was further reduced after 24 h exposure. The same response was seen for the Cftr activity in Ussing chamber measurements in which the forskolin-induced and glibenclamide-sensitive I_{SC} were reduced by 65% within 24 h of dexamethasone exposure. Cftr activity further decreased with prolonged dexamethasone treatment reaching a reduction of almost 90% after 72 h.

As shown previously, Cftr expression is developmentally regulated and is greatest during the first and second trimesters [14]. During the canalicular stage of lung development when differentiation into ATI and ATII cells occurs, Cftr expression begins to gradually decline and remains low until birth [14]. Fetal cortisol levels start to rise at the 20–24th week of gestation (canalicular stage) and gradually increase thereafter with a surge prior to labor [26]. In our experiments with FDLE cells, Cftr activity is rather small, however FDLE cells are differentiated ATII cells from the late canalicular / early saccular stage of lung development, when a decline of Cftr presumably has already occurred in the fetus and Cftr activity is still strongly responsive to GC exposure. We therefore propose that the rise of fetal cortisol levels during the late canalicular stage of lung development is responsible for the decline of CFTR expression observed during this stage. In adult rat ATII cells Cftr mRNA expression and activity were affected in the same manner as in FDLE cells. Therefore, the effect of GCs does not depend on the developmental stage of the cells. In human alveolar A549 cells, dexamethasone also reduced CFTR mRNA expression by more than 80% showing that the effect of GCs is not species-specific as human and rodent alveolar cells reacted similarly. Taken together, GCs strongly antagonize CFTR/Cftr expression and activity in cells of alveolar origin, which possibly represents an important mechanism in the transition from fluid secretion to absorption in fetal lung development. It is known that GCs stimulate absorption by increasing the Na^+ transport via ENaC [17,18]; however, the notion that GCs also reduce secretion by inhibiting CFTR has not been suggested before.

Studies addressing the stimulation of lung growth possibly by enhancing epithelial Cl^- secretion could be valuable in circumstances associated with pulmonary hypoplasia as congenital diaphragmatic hernia (CDH) or prolonged oligohydramnios [27]. Therefore, knowledge of the hormonal regulation of pulmonary Cl^- secretion is an important step in understanding and possibly influencing fetal lung growth. A line of evidence suggests that CFTR is indeed involved in prenatal Cl^- secretion. First of all the expression pattern of CFTR suggests an involvement in fetal lung development. In fact, it has been shown that Cftr over-expression during lung development increases epithelial cell proliferation and enhances secretory cell differentiation [6]. Criticism was raised against this hypothesis due to the lack of overt pathological changes in the CF fetus, however data suggests that early functional changes are present in the lungs of CF patients despite the lack of observable clinical pathology [28–31]. CDH lungs exhibit epithelial cell immaturity supposedly resulting from an impaired branching morphogenesis in early lung development [32]. In a nitrofen-treated CDH rat model, Larson and Cohen, 2006 analyzed whether Cftr over-expression during fetal development would improve the phenotype. The treatment enhanced internal surface area, saccular density, saccular number and amount of saccular air spaces in the lungs suggesting an acceleration of lung development by Cftr over-

expression in the CDH model [7]. It was suggested that this effect was achieved by enhancing lung fluid secretion and consecutively the tension of lung tissue [33]. These studies therefore suggest that CFTR is involved in prenatal Cl^- secretion and thereby lung proliferation.

Near term, the rate of lung fluid secretion and the volume of fetal lung fluid decrease [27,34]. A slowing of fluid production in response to cortisol was reported in fetal guinea pigs, which occurred in addition to an increase of fluid absorption [35]. GCs are further implicated to inhibit lung growth in favor of differentiation [36]. More precisely, cortisol decreases fetal pulmonary cellular growth in early gestation whilst enhancing maturation and slowing growth as term approaches [37]. In fetal lung explants of the pseudoglandular stage it was demonstrated that treatment with dexamethasone distorted the overall branching pattern and resulted in a decreased proliferation of distal epithelial cells [38]. Furthermore, lung specific knock-out of the GR in mice results in a marked hypercellularity [39]. The morphological changes were the result of continued cell division in the distal and proximal epithelia and were attributed to an increased cell proliferation [39]. This suggests that if the lung is not responsive to GCs the epithelial proliferation proceeds unimpeded. It is known that growth arrest is a prerequisite to the induction for terminal differentiation of cells [40] which is mediated by GCs in the lung. Therefore the developmental effects of GCs on fetal lung growth are well known, but that this effect might be mediated by a modulation of CFTR expression has not yet been suggested. Another physiological function of the CFTR decline during fetal lung development could be to enable ENaC activity since CFTR is known to inhibit ENaC [41]. Moreover, ENaC mRNA expression is inversely proportional to the levels of Cftr mRNA in the developing lung suggesting a coordinated regulation [14]. Thus a decrease of CFTR would diminish ENaC inhibition and thereby further increase ENaC mediated Na^+ absorption and perinatal fluid clearance in alveolar cells.

Since dexamethasone reduced CFTR/Cftr mRNA expression in alveolar cells it is surprising that inhibition of the GR by mifepristone alone also reduced Cftr expression. On the other hand, dexamethasone did not further decrease Cftr expression when mifepristone was present in the culture media. Therefore inhibition of the GR and incubation with dexamethasone both decrease Cftr expression. It appears that a minimal function of the GR may be necessary to optimize Cftr expression. Besides, mifepristone is also known to inhibit the progesterone receptor [42], which could also account for the reduced Cftr expression level induced by mifepristone. However, Cftr activity in Ussing chambers was not affected by mifepristone and Cftr activity was restored to control levels in the presence of dexamethasone.

Measurements in human bronchial submucosal gland-derived Calu-3 cells showed a reduction of CFTR mRNA expression by approximately 20% induced by 100 nM dexamethasone. In accordance to our results, Prota and colleagues, 2012 reported that dexamethasone reduced CFTR mRNA expression in Calu-3 cells by 30% [43]. The same study showed that dexamethasone increased CFTR protein expression two-fold which was attributed to an altered chaperone interaction with CFTR resulting in increased protein trafficking [43]. We were able to confirm the reduction of mRNA expression in Calu-3 cells and, in addition, demonstrated an increased CFTR activity in Ussing chamber measurements. CFTR activity was increased to 130% as indicated by the forskolin-induced I_{SC} and to approximately 180% as indicated by the glibenclamide-sensitive I_{SC}. Furthermore, no increase of forskolin-induced I_{SC} by dexamethasone was detected when Cl^- was omitted in the Ussing chamber solution. By contrast, measurements in HCO_3^- free solution resulted in a significantly increased forskolin-induced I_{SC} stimulated by dexamethasone. Therefore, dexamethasone seems to increase only the Cl^- transport in Calu-3 cells. Different aspects might explain the discrepancy between forskolin-induced HCO_3^- and Cl^- transport. First, CFTR has a HCO_3^- permeability of around 20% compared to Cl^- [44], so differences in CFTR HCO_3^- conductance might not be easily detectable. Furthermore, Calu-3 cells apically express a Cl^-/HCO_3^- exchanger of the SLC26A family which is also stimulated by

forskolin [45]. It is unknown if this anion exchanger is affected by GCs, but its activity might contribute to the measured current, as it also secretes HCO_3^- into the apical lumen. In addition, some studies suggested that CFTR displays a "dynamic" selectivity and can switch between Cl^- permeable and HCO_3^-/Cl^- permeable states [46,47]. This is interesting since one study showed that glutamate exclusively elicits Cl^-, but not HCO_3^- conductance in the human sweat duct [46]. However, these results are controversial [48] and it is not known if GCs stimulate a "dynamic" selectivity. Finally, Cl^- entry into the cells might also affect Cl^- secretion and it is yet unknown if GCs alter apical or basolateral Cl^- or HCO_3^- entry pathways.

In accordance to results obtained with Calu-3 cells, CFTR mRNA expression was also reduced by GCs in human bronchial epithelial 16HBE14o- cells, resulting in a 50% lower CFTR expression. Furthermore, treatment of primary rat airway epithelial cells with dexamethasone reduced Cftr mRNA expression by approximately 60% and increased CFTR activity to 150% for the forskolin-induced I_{SC} and 181% for the $CFTR_{172}$inh-sensitive I_{SC}. Therefore, the response to GCs seems to be conserved among airway-derived and alveolar cells.

Inhibition of the GR by mifepristone slightly reduced CFTR mRNA expression in Calu-3 cells but not to a significant extent. Furthermore, dexamethasone did not reduce CFTR expression in the presence of mifepristone. Therefore, GR inhibition itself has a small effect on CFTR expression but prevents the CFTR reduction induced by dexamethasone. Mifepristone also prevented the dexamethasone-induced increase of CFTR activity. Therefore, both the decrease of CFTR mRNA expression and the increase of CFTR activity induced by dexamethasone are mediated by the GR. In agreement with these results a study reported that the dexamethasone-induced occupancy of multiple GR elements causes the repression of CFTR expression in 16HBE14o- cells [49].

In addition, LY-294002 also prevented the increase of CFTR activity induced by dexamethasone because no difference was observed between LY-294002-treated and LY-294002/dexamethasone-treated Calu-3 cells. LY-294002 also strongly reduced basal CFTR activity suggesting that the PI3K is a major regulator of CFTR activity. LY-294002 has been reported to inhibit the forskolin-induced phosphorylation of CFTR by protein kinase A and C and the forskolin-stimulated CFTR trafficking to the plasma membrane of duodenal epithelial cells [50]. This might explain the CFTR current reduction observed in the presence of LY-294002 in the basal as well as dexamethasone-stimulated I_{SC}. In addition to the PI3K, the serum and glucocorticoid-dependent kinase 1 (SGK1) increases Na^+ absorption by modulating the inhibition of ENaC by the ubiquitin ligase neural precursor cell expressed, developmentally down-regulated 4–2 (Nedd4-2) [51]. The same mechanism was proposed for CFTR as SGK1 enhances the functional activity of CFTR when coexpressed in *Xenopus* oocytes [52,53]. Studies further demonstrated that dexamethasone elevates the functional expression of wildtype (wt)- and ΔF508-CFTR and inhibition of either GR or PI3K and knock-down of SGK1 blocks the stimulating effect of dexamethasone on CFTR trafficking in pancreatic cells [54]. Moreover, in the transformed CF bronchial epithelial cell line CFBE41o- the GC-induced increase of SGK1 protein abundance enhanced ΔF508-CFTR [55] and wt-CFTR membrane abundance by inhibiting their endocytic retrieval [56]. CFTR was also shown to be involved in the regulation of stretch-induced proliferation in the lung by controlling airway smooth muscle contractions [57]. The effect of CFTR on muscle contraction was blocked by LY-294002 suggesting that CFTR-dependent proliferation proceeds by a pathway dependent on PI3K and protein kinase CK2 (also known as casein kinase 2) [57]. Therefore our results confirm a critical involvement of the PI3K in the regulation of CFTR activity. However the question remains why this effect on CFTR observed in airway-derived cells is not detected in alveolar cells. The reason for this might be the massive reduction of CFTR/Cftr mRNA expression in alveolar cells compared to the smaller response of bronchial CFTR expression that even a stimulating effect of GC possibly mediated by the PI3K in alveolar cells cannot

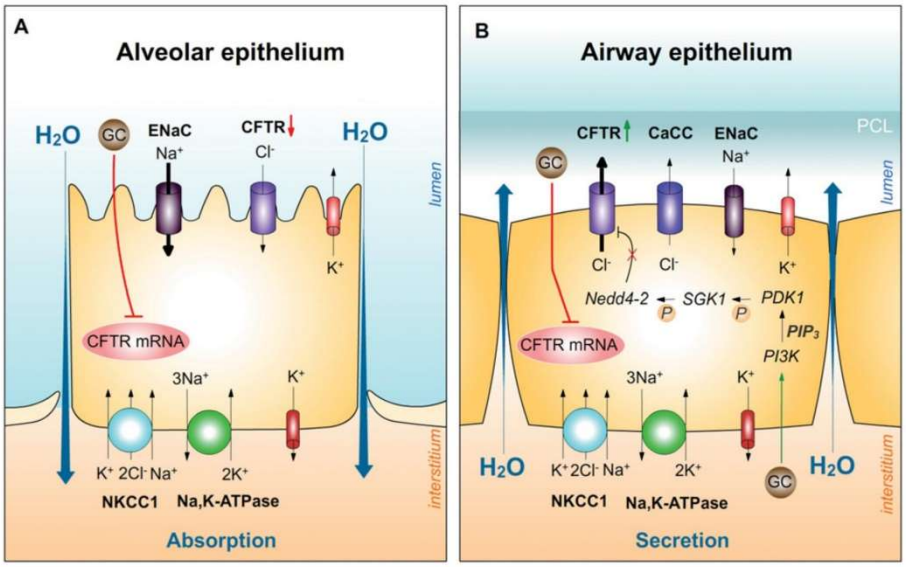

Fig 11. Effect of GCs on CFTR expression and activity in alveolar and airway epithelial cells. A: Postnatal alveolar cells predominantly exhibit Na^+ absorption. Na^+ enters the alveolar cells through apical Na^+ channels like ENaC and is actively extruded basolaterally by the Na,K-ATPase. Thereby an osmotic gradient is generated that drives fluid absorption from the alveolar lumen into the interstitium. Apical CFTR is suggested to mainly absorb Cl^- in postnatal alveolar cells. GCs were shown to markedly reduce CFTR mRNA expression and activity in alveolar cells. **B:** In airway epithelial cells, Cl^- enters the basolateral membrane across NKCC1 and Na^+ is actively extruded by the Na,K-ATPase. K^+ channels recycle K^+ at the basolateral side hyperpolarizing the membrane which represents the driving force for apical Cl^- extrusion through CFTR and Ca^{2+}-dependent Cl^- channels (CaCC). Vectorial Cl^- transport drives water secretion into the airways. GCs reduced CFTR mRNA expression, but increased CFTR activity which supposedly depends on the proposed kinase pathway. GCs activate the PI3K leading to the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Phosphoinositide-dependent kinase-1 (PDK1) is activated and further phosphorylates and activates SGK1. SGK1 in turn interacts with the ubiquitin-ligase Nedd4-2 reducing the affinity of Nedd4-2 for CFTR. Thereby the endocytic retrieval of CFTR is inhibited and CFTR plasma membrane abundance increased. PCL = periciliary liquid layer. Not all potentially important ion transporters and kinases are included in this schematic model.

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measurably increase CFTR activity. Fig 11 summarizes the observed effects of GCs on CFTR/Cftr expression and activity in alveolar and airway-derived cells and proposes a potential pathway leading to an increased CFTR/Cftr activity in airway cells involving the kinases discussed above.

Finally we analyzed the impact of GCs on alternative Cl^- transporters in alveolar and bronchial cells. ANO1 dominates the Ca^{2+}-dependent Cl^- currents in pulmonary epithelia and is expressed at the apical membrane next to CFTR [58]. ANO1/Ano1 mRNA expression was strongly reduced by dexamethasone in alveolar cells whereas ANO1 expression was increased in bronchial 16HBE14o- and Calu-3 cells. The reason for this differential regulation of ANO1/Ano1 by GCs is yet unknown. We suggest that the relevance of this effect might be due to the physiological function of the analyzed cells. During and after birth the main task of alveolar cells is the absorption of fluid to promote air breathing while cells of the bronchial glands play a major role in secretion of the airway lining fluid. If ANO1/Ano1 participates in Cl^- secretion the reduction in alveolar cells and the increase in bronchial cells induced by GCs possibly promotes the physiological task of both cell types. It is unknown if ANO1 is developmentally regulated in the same manner as CFTR, yet the demonstrated hormonal regulation of ANO1/Ano1 suggests a physiological function during development. In addition, Ano1 knock-out mice display a pronounced tracheomalacia, accumulate mucous in the tracheal lumen and die during the early

postnatal period [59,60] further delineating an important function of ANO1 during fetal development. CLC5 and ANO6 are described as components of the outwardly rectifying Cl^- channels (ORCC) [58,61,62]. Both ANO6/Ano6 and CLC5/Clc5 mRNA expression were up-regulated by dexamethasone in alveolar cells, whereas in bronchial 16HBE14o- and Calu-3 cells the expression of the ORCC components was not affected. The anoctamine family consists of at least 10 members and it is known that in addition to ANO1 and ANO6, ANO2 and ANO7 show Cl^- channel activity (see review [63]). A hormonal regulation of ANO2 and ANO7 has yet to be determined. Finally, NKCC1/Nkcc1 mRNA expression was strongly reduced by dexamethasone in alveolar and also in bronchial cells. Since it has been shown that prenatal fluid and Cl^- secretion is dependent on NKCC1 function, the results demonstrate a reduction of secretion possibly to enable perinatal fluid absorption.

Taken together the results show a differential regulation of CFTR/Cftr by GCs (Fig 11). CFTR/Cftr mRNA expression was reduced in alveolar and bronchial cells yet not to the same extent and CFTR/Cftr expression in alveolar cells is more responsive to GC treatment. The reason for this is yet unknown but postnatal alveolar cells are mainly Na^+ absorptive whereas airway cells still secrete Cl^-. The cause for the differential effect of GC on CFTR/Cftr channel activity has yet to be determined but transcriptional and post-translational effects seem to play a part. In summary, the study provides three conclusions. The first conclusion proposes a physiological correlation between the GC concentration and CFTR expression and function in the fetus and thereby possibly marks the switch from lung proliferation to epithelial differentiation. The second conclusion suggests that GCs not only increase absorption during perinatal lung transition but simultaneously diminish secretion by reducing alveolar CFTR. The third conclusion proposes that the CFTR reduction induced by GCs further enables ENaC activity and thereby enhances perinatal fluid absorption.

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Author Contributions

Conceived and designed the experiments: ML UHT. Performed the experiments: ML MB. Analyzed the data: ML MB UHT. Contributed reagents/materials/analysis tools: ML UHT. Wrote the paper: ML UHT.

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5.2

Signaling Cascade involved in Rapid Stimulation of Cystic Fibrosis Conductance Regulator (CFTR) by Dexamethasone

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Signaling Cascade Involved in Rapid Stimulation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by Dexamethasone

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Abstract: Impairment of mucociliary clearance with reduced airway fluid secretion leads to chronically inflamed airways. Cystic fibrosis transmembrane conductance regulator (CFTR) is crucially involved in airway fluid secretion and dexamethasone (dexa) has previously been shown to elevate CFTR activity in airway epithelial cells. However, the pathway by which dexa increases CFTR activity is largely unknown. We aimed to determine whether the increase of CFTR activity by dexa is achieved by non-genomic signaling and hypothesized that the phosphoinositide 3-kinase (PI3K) pathway is involved in CFTR stimulation. Primary rat airway epithelial cells and human bronchial submucosal gland-derived Calu-3 cells were analyzed in Ussing chambers and kinase activation was determined by Western blots. Results demonstrated a critical involvement of PI3K and protein kinase B (AKT) signaling in the dexa-induced increase of CFTR activity, while serum and glucocorticoid dependent kinase 1 (SGK1) activity was not essential. We further demonstrated a reduced neural precursor cell expressed, developmentally downregulated 4-like (NEDD4L) ubiquitin E3 ligase activity induced by dexa, possibly responsible for the elevated CFTR activity. Finally, increases of CFTR activity by dexa were demonstrated within 30 min accompanied by rapid activation of AKT. In conclusion, dexa induces a rapid stimulation of CFTR activity which depends on PI3K/AKT signaling in airway epithelial cells. Glucocorticoids might thus represent, in addition to their immunomodulatory actions, a therapeutic strategy to rapidly increase airway fluid secretion.

Keywords: cystic fibrosis transmembrane conductance regulator; glucocorticoids; dexamethasone; airway; phosphoinositide 3-kinase; serum and glucocorticoid dependent kinase 1; protein kinase B; neural precursor cell expressed, developmentally downregulated 4-like

1. Introduction

Glucocorticoids (GCs) exert many physiological functions including regulation of pulmonary ion channels. The classical genomic mechanism of GC action is mediated by the cytosolic glucocorticoid receptor (GR). Different GR isoforms are produced by alternative splicing. In respiratory epithelial cells, human glucocorticoid receptor-α (hGR-α) is the predominant isoform, displaying steroid-binding activity. In contrast, hGR-β does not show ligand-binding activity [1] and inhibits the transcriptional activity of hGR-α by formation of transcription impairing hGR-α/hGR-β heterodimers [2]. Non-genomic effects of GC can also be mediated by proteins that dissociate from the cytosolic GR-multiprotein complex following GC binding [3]. In addition, rapid GC effects can be mediated by nonspecific interactions of GCs with cellular membranes and specific interactions with membrane-bound GRs [3].

Fluid homeostasis within the conducting segments of the airways is achieved by controlled lung fluid production and absorption, mediated by ion channels like the cystic fibrosis transmembrane conductance regulator (CFTR). Disturbances can affect mucociliary clearance (MCC), which is based on the correct height and thickness of the periciliary liquid layer [4,5]. In the case of dysfunctional CFTR, MCC is impaired, leading to chronically inflamed airways [6]. CFTR dysfunctions may develop due to environmental toxins, cigarette smoke, genetic diseases like cystic fibrosis or acquired pathologies like chronic obstructive pulmonary disease (COPD) [7,8]. Therefore, determining GC effects on the secretion process in the upper airways in relation to MCC is important since anti-inflammatory therapies with GCs are common for chronic airway inflammatory conditions, such as infections and allergies.

CFTR is a plasma membrane cyclic AMP (cAMP)-regulated chloride (Cl^-) channel located at the apical membrane of epithelial cells in several tissues. To date, little is known about the effect of hormones on CFTR function, which appears to differ between the distal and the proximal airways. Our previous study showed that GCs reduced CFTR expression and activity in distal lung epithelial cells, which are responsible for gas exchange and fluid absorption [9]. In contrast, GCs stimulated CFTR activity in the air conducting proximal airways, which are responsible for MCC. Although GCs increased CFTR activity, its gene expression was also reduced, yet not as pronounced as in the distal epithelia. These results led us to address the pathway that results in an increased CFTR activity in bronchial/tracheal epithelia. Since GCs negatively regulated CFTR gene expression, non-genomic pathways were assumed. In agreement, GCs have been shown to induce effects within minutes, incompatible with effects on gene transcription by GRs [10–12]. Supporting our hypothesis, studies showed that GCs increased CFTR protein expression two-fold, which was attributed to an altered chaperone interaction resulting in increased CFTR protein trafficking [13]. Moreover, in the transformed cystic fibrosis (CF) bronchial epithelial cell line CFBE41o-, the GC-induced increase of serum and GC dependent kinase 1 (SGK1) protein abundance enhanced ΔF508-CFTR [14] and wildtype (wt) CFTR membrane expression by inhibiting their endocytic retrieval [15]. SGK1 phosphorylates and thereby inhibits the ubiquitin ligase neural precursor cell expressed, developmentally down-regulated protein 4-like (NEDD4L), a mechanism possibly responsible for the elevated CFTR protein abundance. These studies in combination with our previous results suggest that kinase signaling is majorly involved in stimulated CFTR activity induced by GCs in airway epithelial cells. We thus aimed to determine signaling pathways leading to stimulation of CFTR activity by GCs using dexamethasone (dexa) in primary rat airway epithelial cells and human bronchial epithelial Calu-3 cells.

2. Results

2.1. The Phosphoinositide 3-Kinase (PI3K) Pathway Is Involved in the Dexa-Stimulated Cystic Fibrosis Transmembrane Conductance Regulator Activity

To determine the signaling pathway of dexa action we used different kinase inhibitors. First, dexa increased CFTR activity in Ussing chamber measurements, as demonstrated by the significantly elevated $CFTR_{inh}172$-sensitive I_{SC}, in primary airway epithelial cells ($p < 0.01$, Figure 1). LY-294002 was used to block the phosphoinositide 3-kinase (PI3K) and measurements showed that dexa was unable to increase CFTR activity when LY-294002 was present ($p < 0.001$). These experiments showed that the PI3K activity is indispensable for the stimulating effect of dexa on CFTR activity.

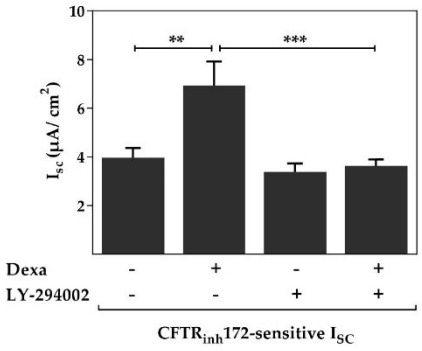

Figure 1. Phosphoinositide 3-kinase (PI3K) contributes to the increased cystic fibrosis transmembrane conductance regulator (CFTR) activity induced by dexa. Primary airway epithelial cells were treated with 100 nM dexa and LY-294002 (10 µM) for 24 h. Data bars represent mean + standard error of the mean (SEM) of I_{SC}. Dexa increased the $CFTR_{inh}$172-sensitive I_{SC}. Addition of LY-294002 prevented the dexa-induced increase of CFTR activity (n = 20–25, ** $p < 0.01$, *** $p < 0.001$, analysis of variance (ANOVA) with Tukey's *post hoc* test).

2.2. Serum and Glucocorticoid Dependent Kinase 1 Is Not Involved in the Dexa-Stimulated CFTR Activity

In contrast to the contribution of PI3K, the inhibitor of SGK1, GSK650394 had no effect on the dexa-stimulated CFTR activity in either primary airway epithelial or Calu-3 cells. Dexa was still able to significantly increase CFTR activity even in the presence of GSK650394 ($p < 0.05$, $p < 0.01$, Figure 2a,b).

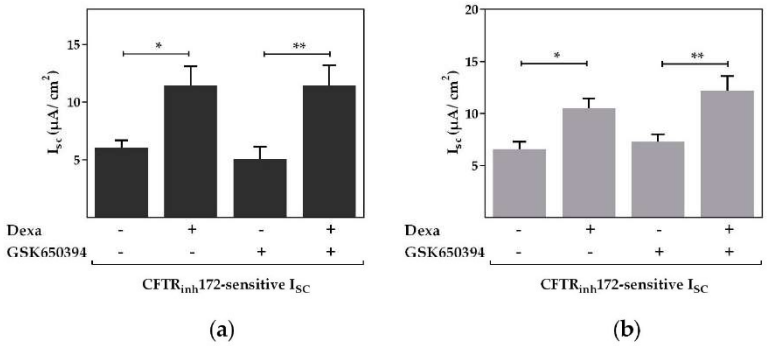

Figure 2. Serum and glucocorticoid dependent kinase 1 activity is not involved in the dexa-stimulated CFTR activity. Cells were treated with 100 nM dexa and GSK650394 (10 µM) for 24 h. Data bars represent mean + SEM of I_{SC}. (**a**) In primary airway epithelial cells, the $CFTR_{inh}$172-sensitive I_{SC} was increased by dexa. Addition of GSK650394 displayed no effect on the dexa-induced increase of CFTR activity (n = 10–15, * $p < 0.05$, ** $p < 0.01$, ANOVA with Tukey's *post hoc* test); (**b**) in Calu-3 cells $CFTR_{inh}$172-sensitive I_{SC} was increased by dexa. In agreement, GSK650394 displayed no effect on the dexa-induced increase of CFTR activity (n = 24–31, * $p < 0.05$, ** $p < 0.01$, ANOVA with Tukey's *post hoc* test).

It is known that SGK1 activity is elevated by GC exposure. Western blot measurements showed that dexa significantly increased phosphorylation of n-myc downregulated gene 1 (NDRG1), a specific substrate of SGK1, and thus representing an elevated SGK1 enzyme activity compared with control cells

($p < 0.05$, Figure 3a). Therefore, the results do support an activation of SGK1 by dexa. However, both the addition of mifepristone to inhibit the GR and GSK650394 suppressed the increased SGK1 activity induced by dexa, as shown by a significant reduction of phosphorylated NDRG1, which reached unstimulated control levels ($p < 0.05$, Figure 3a,b). The Western blot measurement therefore shows that dexa increases SGK1 activity and that GSK650394 is effective in blocking SGK1 activation by dexa. Since the effect of dexa in Ussing chamber measurements persisted after the application of GSK650394, the activity of SGK1 is not decisively involved in dexa-stimulated CFTR activity.

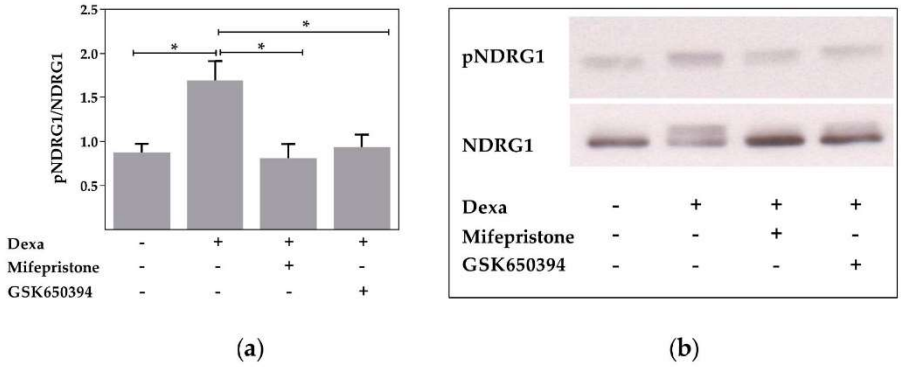

Figure 3. SGK1 activity is elevated by dexa and reduced by GSK650394. (**a**) Normalized densitometric evaluation of n-myc downregulated gene 1 (NDRG1) and pNDRG1 Western blots. Calu-3 cells were treated with 100 nM dexa and GSK650394 (10 μM) or mifepristone (10 μM) for 24 h ($n = 4$; * $p < 0.05$ by *t*-test); (**b**) western blot of pNDRG1 and total NDRG1 resulted in bands of 46 and 48 kDa.

2.3. Protein Kinase B Activity Is Involved in the Dexa-Stimulated CFTR Activity

PI3K signaling affects ion channel activity through SGK1 and/or protein kinase B (AKT). We thus checked for an involvement of AKT in the dexa-stimulated CFTR activity. Akt1/2 kinase inhibitor was used to block AKT activation which decreased CFTR activity in control and dexa-stimulated cells. Similar results were obtained in primary airway epithelial and Calu-3 cells ($p < 0.05$, $p < 0.001$, Figure 4a,b). Furthermore, dexa was unable to increase CFTR activity when Akt1/2 kinase inhibitor was present. Therefore, in addition to PI3K, AKT is indispensable for enhancement of CFTR activity by dexa.

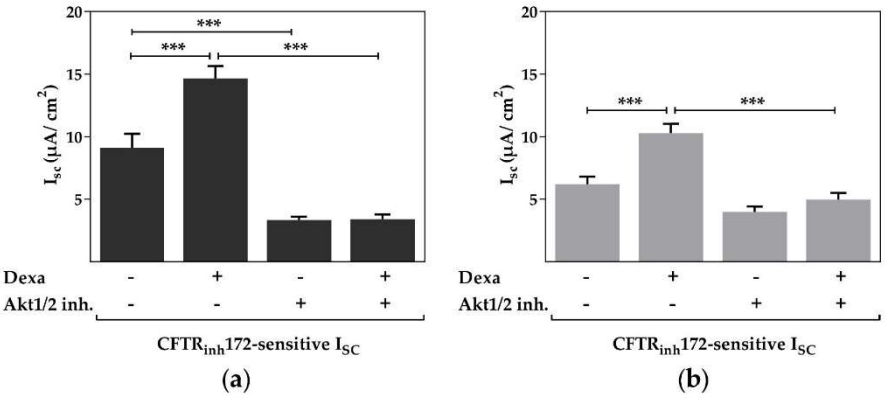

Figure 4. Akt1/2 kinase inhibitor prevents the increase of CFTR activity induced by dexa. Cells were treated with 100 nM dexa and Akt1/2 kinase inhibitor (10 µM) for 24 h. Data bars represent the mean + SEM of I_{SC}. (**a**) In primary airway epithelial cells, the CFTR$_{inh}$172-sensitive I_{SC} was increased by dexa. Addition of Akt1/2 kinase inhibitor prevented the dexa-induced increase of CFTR activity (n = 11–14; *** $p < 0.001$, ANOVA with Tukey's *post hoc* test); (**b**) in Calu-3 cells the CFTR$_{inh}$172-sensitive I_{SC} was increased by dexa. Likewise, addition of Akt1/2 kinase inhibitor prevented the dexa-induced increase of CFTR activity (n = 36–37, *** $p < 0.001$, ANOVA with Tukey's *post hoc* test).

To verify an involvement of AKT in the stimulation by dexa, we analyzed the phosphorylation of AKT with Western blot. Western blot measurements showed that dexa significantly increased phosphorylation of AKT at Ser473, representing an elevated AKT enzyme activity ($p < 0.05$, Figure 5a,b). Both the addition of mifepristone and Akt1/2 kinase inhibitor blocked the increased AKT activity induced by dexa, as shown by a significant reduction of phosphorylated AKT, which reached unstimulated control levels ($p < 0.05$, $p < 0.001$, Figure 5a,b). The Western blot measurement therefore shows that dexa increases AKT activity and that both, Akt1/2 kinase inhibitor and GR inhibition are effective in blocking AKT activation by dexa.

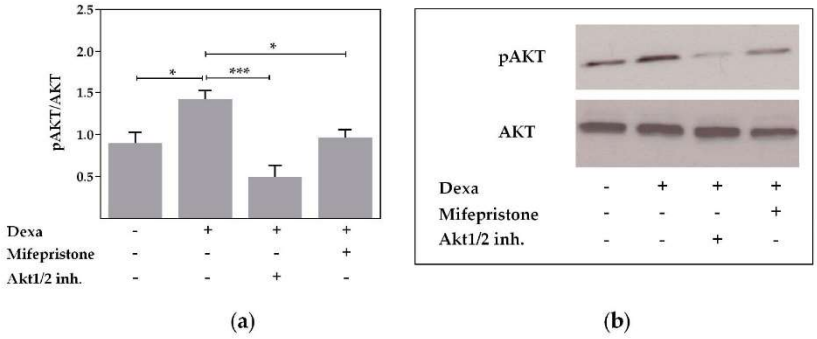

Figure 5. AKT activity is elevated by dexa. (**a**) Normalized densitometric evaluation of pAKT and AKT Western blots. Calu-3 cells were treated with 100 nM dexa and Akt1/2 kinase inhibitor (10 µM) or mifepristone (10 µM) for 24 h. Addition of mifepristone and Akt1/2 kinase inhibitor blocked the increased AKT activity induced by dexa (n = 4; * $p < 0.05$; *** $p < 0.001$ by t-test); (**b**) western blot of pAKT and total AKT resulted in bands of 60 kDa.

2.4. Neural Precursor Cell Expressed, Developmentally Downregulated 4-Like Activity Is Reduced by Dexa

NEDD4L degrades membrane-bound ion channels and thereby decreases channel activity. NEDD4L activity can be affected by phosphorylation through AKT or SGK1 that reduces its enzyme activity. We thus analyzed if phosphorylation of NEDD4L is affected by dexa in Calu-3 cells and found phosphorylation of NEDD4L to be significantly increased by dexa ($p < 0.05$, Figure 6a–d). Mifepristone prevented the dexa-stimulated increase of phosphorylated NEDD4L ($p < 0.05$, Figure 6a,b). Furthermore, AKT inhibition by Akt1/2 kinase inhibitor reduced phosphorylation of NEDD4L in dexa-stimulated cells, which reached unstimulated control levels ($p < 0.05$, Figure 6c,d). In agreement with our previous results, SGK1 inhibition by GSK650394 had no significant effect on phosphorylated NEDD4L in dexa-stimulated cells (Figure 6c,d). In conclusion, the Western blots show that dexa decreases NEDD4L activity by increasing its phosphorylation, which is blocked by GR and AKT inhibition.

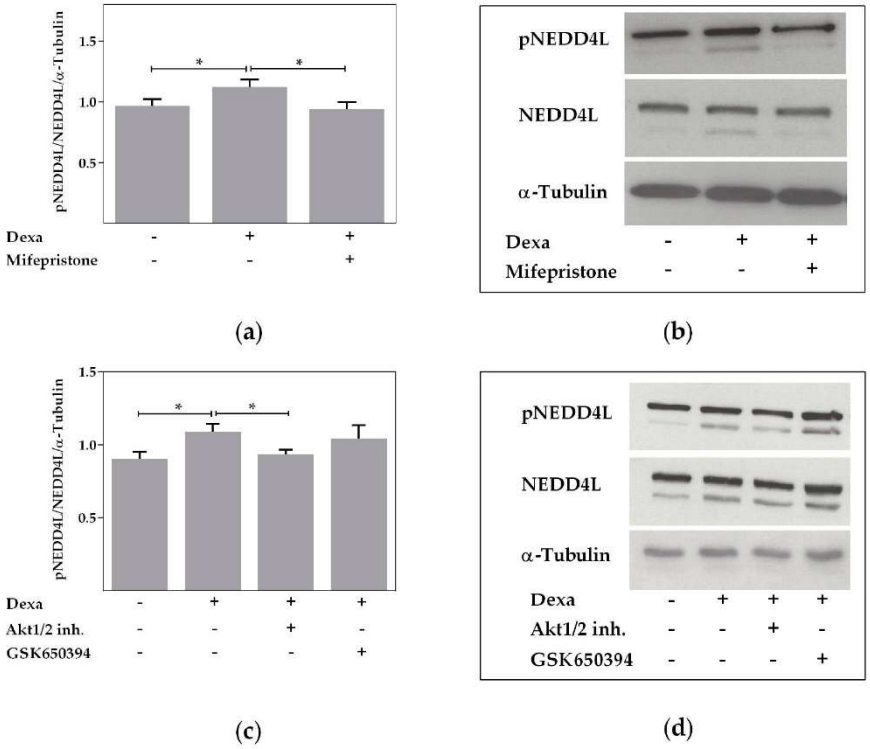

Figure 6. Dexa increases phosphorylation of neural precursor cell expressed, developmentally downregulated 4-like (NEDD4L). (**a**) Normalized densitometric evaluation of pNEDD4L, NEDD4L and α-tubulin Western blots. Calu-3 cells were treated with 100 nM dexa and mifepristone (10 μM) for 24 h ($n = 5$; * $p < 0.05$ by t-test); (**b**) western blot of pNEDD4L and total NEDD4L resulted in bands of 110 and 135 kDa. Western blot of α-tubulin resulted in bands of 52 kDa; (**c**) normalized densitometric evaluation of pNEDD4L, NEDD4L and α-tubulin Western blots. Calu-3 cells were treated with 100 nM dexa and Akt1/2 kinase inhibitor (10 μM) or GSK650394 (10 μM) for 24 h ($n = 5$; * $p < 0.05$ by t-test); (**d**) western blot of pNEDD4L and total NEDD4L resulted in bands of 110 and 135 kDa. Western blot of α-tubulin resulted in bands of 52 kDa.

2.5. Rapid Effects of Dexa on CFTR Activity

The described signaling pathway of dexa should be able to render its effect within very short time frames. To test this hypothesis, we stimulated Calu-3 cells with dexa for only 30 min instead of 24 h. Even within this short time frame, CFTR activity was significantly increased by dexa as demonstrated in Ussing chamber measurements ($p < 0.05$, Figure 7a). On the other hand, CFTR mRNA expression was unaffected after short term exposure to dexa (Figure 7b). Finally, phosphorylation of AKT, NDRG1 and NEDD4L was demonstrated within 30 min of dexa application ($p < 0.05$, Figure 7c–e), further supporting a rapid kinase-dependent signaling pathway of dexa stimulation.

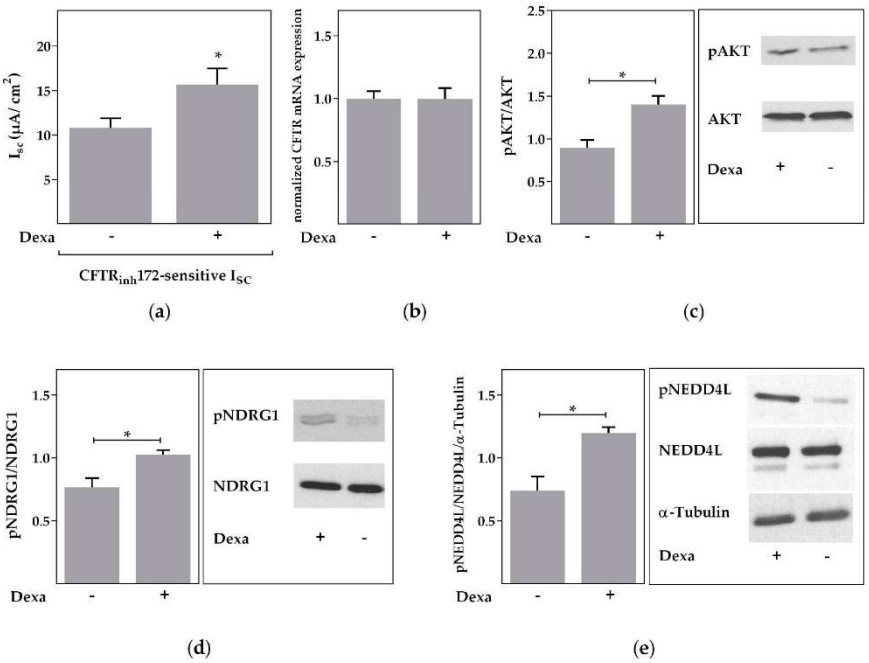

Figure 7. Rapid effects of dexa on CFTR activity. Calu-3 cells were treated with 100 nM dexa for 30 min. Data bars represent the mean + SEM. (**a**) The CFTR$_{inh}$172-sensitive I_{SC} was significantly increased by dexa ($n = 60$, * $p < 0.05$ by t-test with Welch's correction); (**b**) CFTR mRNA expression was not affected by dexa ($n = 6$); (**c**) normalized densitometric evaluation of pAKT and AKT Western blots ($n = 3$; * $p < 0.05$ by t-test). Western blot of pAKT and total AKT resulted in bands of 60 kDa; (**d**) normalized densitometric evaluation of pNDRG1 and NDRG1 Western blots ($n = 3$; * $p < 0.05$ by t-test). Western blot of pNDRG1 and total NDRG1 resulted in bands of 46 and 48 kDa; (**e**) normalized densitometric evaluation of pNEDD4L, NEDD4L and α-tubulin Western blots ($n = 3$; * $p < 0.05$ by t-test). Western blot of pNEDD4L and total NEDD4L resulted in bands of 110 and 135 kDa. Western blot of α-tubulin resulted in bands of 52 kDa.

3. Discussion

CFTR channels are critical for airway function and GCs have been shown to stimulate CFTR activity in airway epithelial cells [9]. This is especially true for chloride secretion, and therefore maintenance of airway surface liquid layer is controlled by CFTR. Regarding the broad clinical use of GCs for several airway disorders, it is of central importance to elucidate their impact on epithelial

physiology. Herein, we aimed to determine whether the increase of CFTR activity by dexa is achieved by non-genomic signaling and hypothesized that the PI3K pathway is involved in CFTR stimulation. We demonstrated that PI3K and AKT are indispensable for the stimulatory action of GCs, while SGK1 is not majorly involved in the signaling cascade leading to enhanced CFTR activity. We further showed that GCs increased phosphorylation of AKT, SGK1 and NEDD4L in airway epithelial cells, representing an enhanced activity of AKT and SGK1 and a decreased NEDD4L protein function. AKT inhibition prevented the increased phosphorylation of NEDD4L, while SGK1 inhibition was unable to prevent NEDD4L phosphorylation. Finally, stimulation of CFTR activity by GCs was demonstrated within 30 min, further supporting rapid signaling by kinases.

Our previous study demonstrated that GCs exhibit different effects on proximal and distal airway compartments. Within the proximal air-conducting compartment, GCs promote fluid secretion by enhancing CFTR activity as shown in Calu-3 and primary airway epithelial cells [9]. Airway fluid secretion enhances MCC by maintaining the airway surface liquid layer [4,5]. In accordance with our results, it was shown that dexa increased CFTR protein expression two-fold which was attributed to an altered chaperone interaction resulting in increased CFTR protein trafficking, while CFTR gene expression was also reduced [13]. In distal airways, the absorptive phenotype predominates enabling gas exchange in the alveoli. It might thus make physiological sense that GCs reduced alveolar CFTR activity in distal lung cells, whereas alveolar ENaC activity was increased [16], promoting fluid absorption to ensure efficient gas exchange. Since GCs reduced CFTR gene expression in distal, but also in proximal lung compartments, the stimulation of CFTR activity in airway epithelial cells is most likely mediated by non-genomic mechanisms.

Our results showed that PI3K activity is essential for the stimulatory effect of GCs on CFTR activity in primary airway epithelial cells. Activation of receptor tyrosine kinases results in activation of PI3K. The catalytic domain of PI3K subsequently converts phosphatidylinositol (3,4)-biphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3), leading to activation of phosphoinositide-dependent protein kinase (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), followed by phosphorylation and activation of AKT. Phosphorylation of AKT at Ser473 by mTORC2 facilitates phosphorylation of Thr308 by PDK1 [17]. Inhibition of the PI3K by LY294002 [18], prevented the GC-mediated increase of CFTR activity. Basal CFTR activity was not affected by LY294002 in primary airway epithelial cells, suggesting that PI3K activity is not essential for basal CFTR function in these cells. These results are in contrast to Calu-3 cells in which both the GC-induced and the basal CFTR activity were affected by PI3K inhibition [9]. Differences between human submucosal gland-derived Calu-3 cells and rat primary airway cells might be due to additional mechanisms maintaining basal CFTR activity. Confirming our findings, LY294002 was shown to inhibit the forskolin-induced phosphorylation of CFTR in duodenal epithelial cells as well as CFTR trafficking to the membrane [18]. Further kinase involvement, like that of PIKfyve [19] and CK2 [20] or participation of P2Y2 receptors [21,22] might contribute to maintaining basal CFTR activity, even after PI3K inhibition, in primary airway epithelial cells.

Downstream of PI3K, involvement of SGK1 and AKT in regulation of epithelial ion channels has been reported. SGK1 is a serin/threonine kinase controlled by several stimuli including GCs. SGK1 plays an important role in regulating ion transport as well as cell metabolism and tumor growth, and is activated via PI3K, PDK1 and mTORC2 [23–26]. Nevertheless, Ussing chamber measurements demonstrated that SGK1 inhibition had no effect on CFTR stimulation by GCs in primary airway epithelial and Calu-3 cells, since dexa-stimulated CFTR currents were still elevated even after SGK1 inhibition. Furthermore, basal CFTR activity was not affected by SGK1 inhibition. Western blot analysis confirmed that SGK1 was indeed effectively inhibited by GSK650394, preventing the increased phosphorylation of NDRG1 induced by GCs. In addition, GR inhibition by mifepristone also prevented the increased SGK1 activity induced by GCs. This indicates that SGK1 is not involved in CFTR regulation. In contrast, other studies demonstrated that SGK1 enhances the functional activity of CFTR when coexpressed in *Xenopus* oocytes [26,27]. In pancreatic cells, dexa was shown to elevate

the functional expression of wt- and ΔF508-CFTR by increasing total protein expression, cell surface expression and channel half-life [28]. This study showed that inhibition of either the GR or the PI3K and knock-down of SGK1 blocks the effect of dexa on CFTR trafficking [28]. In agreement with this, the GC-induced increase of SGK1 protein abundance enhanced ΔF508-CFTR [14] and wt-CFTR membrane expression in CFBE41o- cells by inhibiting their endocytic retrieval [15]. Herein, we did not observe a major contribution of SGK1 to the enhanced CFTR activity, although we also did not determine CFTR trafficking or membrane abundance. Therefore, CFTR activity and protein abundance might not be directly comparable. Furthermore, it is known that SGK1 contributes to the stimulating effect of insulin on Na^+ transport. However, in a previous study no major contribution of SGK1 to the stimulating effect of insulin on Na^+ transport was observed in primary alveolar cells [29]. These results were supported by H441 cells, which are of respiratory origin, while in renal mpkCCD cells the same study showed that the impact of insulin was critically dependent on activation of SGK1 [30]. It was therefore suggested that SGK1 exhibits cell type-specific effects [30] or dependence on the presence of local anchoring proteins [31], which might also explain the differing results obtained in our study.

In contrast to SGK1, AKT, another kinase downstream of PI3K, was indispensable for the increased CFTR activity as shown in Ussing chamber measurements. AKT inhibition by Akt1/2 kinase inhibitor markedly decreased CFTR activity in both dexa-stimulated and unstimulated control cells. These results demonstrate that CFTR activity is largely dependent on AKT activity. Furthermore, dexa is unable to stimulate CFTR activity in the presence of Akt1/2 kinase inhibitor. Western blot analysis showed increased Ser473-phosphorylation and thus activation of AKT after incubation with GCs, which was prevented by Akt1/2 kinase inhibitor. Inhibition of the GR by mifepristone reduced AKT phosphorylation accordingly. Akt1/2 kinase inhibitor is a highly selective noncompetitive inhibitor of AKT [32], which prevents the conformational change, triggered by binding of PIP_3 to the pleckstrin homology domain of AKT isoforms, which allows PDK1 and mTORC2 to phosphorylate and activate AKT [33]. However, it has been reported that Akt1/2 kinase inhibitor might also inhibit SGK1, since it was shown to prevent phosphorylation of NDRG1 [34], even though others reported no inhibition of SGK1 with Akt1/2 kinase inhibitor at concentrations as high as 250 μM [32]. In our experiments, we assume that a possible unspecific effect of Akt1/2 kinase inhibitor is negligible, since direct inhibition of SGK1 by GSK650394 did not affect CFTR activity. Therefore, the complete suppression of the GC effect by Akt1/2 kinase inhibitor was attributable to the inhibition of AKT.

NEDD4L ubiquitinates membrane proteins, including ion channels, leading to increased internalization and degradation of its target proteins [35–37]. Western blot analysis demonstrated an increased phosphorylation of NEDD4L induced by dexa in Calu-3 cells. Phosphorylation of NEDD4L at Ser468 increases its binding affinity to the regulatory protein 14-3-3, which in turn suppresses ubiquitin E3 ligase activities of NEDD4L by inhibiting formation of the enzyme/substrate complex [38]. In agreement to Ussing chamber measurements, GR inhibition prevented the effect of dexa on NEDD4L phosphorylation. Studies showed that Na^+ transport is strongly affected by NEDD4L and phosphorylation of NEDD4L thus increases epithelial Na^+ channel (ENaC) membrane abundance by preventing its endocytic retrieval [31,39]. Regarding the relationship between NEDD4L and CFTR, different results were published. In pancreatic CFPAC-1 cells, expression of ΔF508-CFTR was increased by knockdown of NEDD4L [28]. In contrast, neither overexpression nor silencing of NEDD4L affected wt-CFTR apical membrane abundance or Cl^- currents in *Xenopus* oocytes or CFBE-wt cells [40]. However, the authors also suggested that NEDD4L might regulate CFTR indirectly by ubiquitinating positive or negative regulators of CFTR membrane density or activity [40]. More precisely, a small but significant increase in CFTR membrane abundance without a concomitant increase in CFTR Cl^- currents was noted upon NEDD4L overexpression, while CFTR currents increased without elevations in CFTR membrane density with NEDD4L knockdown. The authors suggested that overexpression of NEDD4L might result in increased ubiquitination and degradation of negative regulators of CFTR abundance as well as positive regulators of CFTR activity, resulting in no net increase in CFTR currents. In accordance, NEDD4L knockdown might reduce ubiquitination and degradation of

positive regulators of CFTR activity, enhancing CFTR currents [40]. The proposed suggestion that CFTR protein abundance and channel function might not be directly comparable possibly explains the different results obtained in previously published studies as well as the unaffected CFTR activity by SGK1 inhibition as described above.

Furthermore, AKT and SGK1 are both reported to inhibit the actions of NEDD4L by phosphorylation [31,39,41]. Inhibition of AKT by Akt1/2 kinase inhibitor prevented the elevated NEDD4L phosphorylation induced by dexa, supporting a major involvement of AKT in GC actions. On the other hand, inhibition of SGK1 by GSK650394 had no major effect on NEDD4L phosphorylation, further questioning a contribution of SGK1 in dexa stimulation. Our results therefore support a PI3K/AKT dependent stimulation of CFTR activity by dexa, which is possibly mediated by inhibition of NEDD4L. A direct interaction between CFTR and NEDD4L or NEDD4L and AKT was not determined in this study and must be addressed in future studies. Finally, CFTR activity was significantly increased by dexa within 30 min as demonstrated in Ussing chamber measurements. Interestingly, activation of AKT has been demonstrated within 2 min after GC stimulation in alveolar A-549 cells, which was prevented by PI3K inhibition [10]. We also showed an increased phosphorylation of AKT, NEDD4L and NDRG1 by dexa within 30 min, whereas CFTR mRNA expression was not affected. These results further support the proposed kinase signaling pathway.

Non-genomic effects are generally believed to take place within 30 min of GC application due to the considerable latency of genomic steroid effects [42]. We did not analyze which GR isoform is responsible for the observed effects, but it is important to note that increased hGR-β expression has been correlated with several diseases related to GC resistance [43]. Future studies thus need to characterize the involved GR isoforms and determine whether a short-term GC exposure yielding to rapid increases of CFTR activity harbors a risk for GC-resistant lung disease.

4. Materials and Methods

4.1. Tissue Preparation

All animal care and experimental procedures were approved by the institutional review board (Landesdirektion Leipzig, Permit Number: T36/13, 21th Dec 2012). Sprague-Dawley rats were bred at the Medical Experimental Center (MEZ) of Leipzig University. Animals were housed in rooms with a controlled temperature (22 °C), humidity (55%) and 12 h light-dark cycle. Food and water were freely available. Rats were euthanized by carbon dioxide inhalation.

4.2. Isolation of Primary Airway Epithelial Cells

Isolation of primary airway epithelial cells has been described previously [44]. Briefly, the trachea proximal to the bronchial bifurcation was isolated from euthanized male Sprague-Dawley rats (140–200 g). Esophageal remnants and adherent adipose tissue were removed. The trachea was opened longitudinally and rinsed with Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Darmstadt, Germany) before incubation in DMEM with 0.1% protease XIV (# P5147, Sigma-Aldrich, Munich, Germany), 0.01% DNase I (# D5025, Sigma-Aldrich) and antibiotic-antimycotic (Thermo Fisher Scientific), a mixture of penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL), for 21 h at 4 °C while shaking. The digestion was stopped by the addition of 1 Vol. fetal bovine serum (FBS) (Biochrom, Berlin, Germany). The trachea was then agitated and scraped with a cell scraper to detach the airway epithelial cells. The obtained cell suspension was centrifuged twice at 500× *g* for 5 min and re-suspended in cell culture media. Cell culture media consisted of DMEM-F12 (Thermo Fisher Scientific) with 1 µg/mL insulin (# 91077C, Sigma-Aldrich, Taufkirchen, Germany), 7.5 µg/mL transferrin (# 354204, Corning GmbH, Wiesbaden, Germany), 1 µM hydrocortisone (# H0888, Sigma-Aldrich), 30 nM 3,5,3′-triiodothyronine (# T6397, Sigma-Aldrich), 25 ng/mL epidermal growth factor (# 354052, Corning GmbH), 10 ng/mL endothelial cell growth supplement (# 354006, Corning GmbH), and antibiotic-antimycotic, which was supplemented (1:1)

with 3T3 fibroblast (from ATCC®, Manassas, VA, USA, # CCL-92)-conditioned DMEM containing 2% FBS. Cells were seeded on collagen-coated (human placental collagen type IV, # C5533, Sigma-Aldrich) permeable supports at a density of 2×10^5 cells per Snapwell insert (Costar, # 3407, surface area 1.1 cm^2, Corning GmbH). Medium was changed after the first 24 h and then every two days. Measurements were done approximately 10 days after plating, when the transepithelial resistance (R_{te}) reached values >300 $\Omega \cdot cm^2$. R_{te} was measured during medium change with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) with STX-2 chopstick electrodes. Cells subjected to different experimental conditions were always age matched, treated equally and recorded simultaneously.

4.3. Culture of Cell Lines

Calu-3 cells (from ATCC, # HTB-55), derived from human bronchial submucosal glands, were kindly provided by Getu Abraham (Institute of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, University of Leipzig). Calu-3 cells (passage 23–30) were cultured in DMEM-F12 with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) and 1% non-essential amino acids (Thermo Fisher Scientific), and passaged 1–2 times weekly. Calu-3 cells were seeded at a density of 5×10^5 cells per Snapwell insert and 1×10^6 cells per Transwell insert (Costar, # 3412, surface area 4.6 cm^2). After 10 days, Calu-3 cells were subjected to air-liquid interface conditions and R_{te} was measured every two days during medium change. Measurements and protein isolation were done approximately 14–21 days after plating of Calu-3 cells on permeable supports, when R_{te} reached values >300 $\Omega \cdot cm^2$.

For all analyzed cell types serum-free complete medium (Cellgro, Corning GmbH), supplemented with dexa (100 nM, # D4902, Sigma-Aldrich) was added 24 h before measurement or as stated otherwise. To determine the involvement of the PI3K, the inhibitor LY-294002 (10 µM, # 1130 TOCRIS Bioscience, Bristol, UK) was used. Furthermore, Akt1/2 kinase inhibitor (10 µM, # A6730, Sigma-Aldrich) was used to inhibit the AKT, GSK650394 (10 µM, # 3572, TOCRIS Bioscience) to inhibit the SGK1 and mifepristone (10 µM, # M8046, Sigma-Aldrich) to inhibit the GR.

4.4. Electrophysiological Measurements

A detailed description of Ussing chamber measurement procedures is reported elsewhere [45]. Experiments were included in the data analyses only when R_{te} exceeded 300 $\Omega \cdot cm^2$ throughout the measurement. Ussing chambers were filled with a ringer solution containing: Na^+ 145 mM, K^+ 5 mM, Ca^{2+} 1.2 mM, Mg^{2+} 1.2 mM, Cl^- 125 mM, HCO_3^- 25 mM, $H_2PO_4^-$ 3.3 mM, HPO_4^{2-} 0.8 mM (pH 7.4). The basolateral side contained 10 mM glucose whereas 10 mM mannitol was used in the apical compartment. Equivalent short-circuit currents (I_{SC}) were assessed every 20 s by measuring transepithelial voltage (V_{te}) and R_{te} using a transepithelial current clamp (Physiologic Instruments, San Diego, CA, USA), and calculating the quotient $I_{SC} = V_{te}/R_{te}$. Amiloride (10 µM, # A-7410, Sigma-Aldrich), an inhibitor of ENaC, was added to the apical compartment to inhibit amiloride-sensitive Na^+ channels. Forskolin (10 µM, # F-6886, Sigma-Aldrich) was added to the apical compartment to increase the intracellular cyclic adenosine monophosphate (cAMP) concentration and thereby activate cAMP-sensitive ion channels like CFTR. Finally, $CFTR_{inh}172$ (10 µM, # 3430, TOCRIS Bioscience) was applied apically to determine the $CFTR_{inh}172$-sensitive I_{SC}, a measure of CFTR activity. Amiloride was dissolved in water; forskolin, $CFTR_{inh}172$, mifepristone, GSK650394, Akt1/2 kinase inhibitor and LY-294002 were prepared in dimethyl sulfoxide (DMSO) and dexamethasone in 100% ethanol. Control monolayers were treated with the respective solvent to exclude solvent influence on the evoked responses.

4.5. Western Blot Analyses

Calu-3 cell Transwell inserts were placed on ice, washed with ice-cold phosphate buffered saline solution and overlaid with 250 µL of lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40 substitute, 0.25% sodium deoxycholate,

1 mM Na_3VO_4, 1 mM NaF and the protease inhibitor cocktail Roche complete (Roche GmbH, Mannheim, Germany). After 30 min, filters were excised from their holders and transferred into Eppendorf tubes, carefully collecting all fluid that had flown off. The tubes were freeze-thawed three times, and then centrifuged at 12,000× *g* and 4 °C for 10 min to accumulate the fluid at the bottom of the tubes. The almost dry filters were discarded, leaving the protein-rich lysate. Protein content was measured against bovine serum albumin standards in triplicate by a standard protein assay (BCA, Pierce, Rockford, IL, USA) on 96-well plates with an automatic plate reader. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) through an 8 or 10% gel (40 µg protein per lane) and transferred onto nitrocellulose membrane. Blots were incubated overnight at 4 °C with the following primary antibodies, diluted according to the manufacturer's standard protocol. Phosphorylation of NDRG1 was used to determine SGK1 enzyme activity [30,46,47] and detected with phospho-NDRG1 antibody (# 3217, Cell Signaling Technology, Inc., Danvers, MA, USA), when phosphorylated at Thr346, and total NDRG1 antibody (# 5196, Cell Signaling Technology, Inc.). Phosphorylation of AKT was analyzed using antibodies against phospho-AKT at Ser473 (# 9271, Cell Signaling Technology, Inc.), and AKT (# 9272, Cell Signaling Technology, Inc., both kindly provided by A. Garten). Phosphorylation of NEDD4L was measured with antibodies against phospho-NEDD4L at Ser448 (# 8063, Cell Signaling Technology, Inc.), total NEDD4L (# 4013, Cell Signaling Technology, Inc.) and α-tubulin (# 2144, Cell Signaling Technology, Inc.). Suitable secondary antibodies conjugated to horseradish peroxidase (HRP) were used to detect primary antibodies. HRP activity was analyzed by enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ, USA) on x-ray film and band intensity was measured by densitometry using Image-J (National Institutes of Health (NIH), Bethesda, MD, USA).

4.6. Measurement of CFTR mRNA Expression

Total RNA was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific) and treated with DNase I (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcription was carried out employing the Maxima H Minus DNA Synthesis Kit (ThermoFisher Scientific). The SYBR Select Master Mix (ThermoFisher Scientific) was used for qRT-PCR, following the manufacturer's instructions. Reactions were conducted with the IQ5 rtPCR Detection System (BioRad, Munich, Germany). Transcripts of target genes were amplified using the gene-specific primers for human CFTR 5′-GGGCTGTGTCCTAAGCCATGGCCA-3′ and 5′-GATGGCTTGCCGGAAGAGGCTCC-3′. Absolute quantification was performed using a several fold dilution of target specific plasmid-DNA as internal standard curve. The resulting molecule concentrations were normalized to a reference gene (Mrps18a: mitochondrial ribosomal protein S18a). Constant expression of Mrps18a was confirmed against other common reference genes. The fold change of mRNA levels was calculated with the relative standard curve method. Measurements were performed in technical triplicates and six biological replicates. Melting curves and gel electrophoresis of the PCR products were routinely performed to determine the specifity of the PCR reaction.

4.7. Statistical Analyses

For statistical analyses, GraphPad Prism (version 5.03; GraphPad Software, Inc., San Diego, CA, USA) was used. Differences among groups treated with dexamethasone and controls were evaluated by unpaired *t*-test, or analysis of variance (ANOVA) followed by Tukey's *post hoc* test, as appropriate. A probability of $p < 0.05$ was considered significant for all statistical analyses.

5. Conclusions

The presented results support a rapid, most likely non-genomic signaling pathway of dexa stimulation via PI3K/AKT in airway epithelial cells. Rapid CFTR regulation by GCs might alter the relevance of GCs in acute treatment of airway disorders. Currently, GCs are widely used to treat inflammatory exacerbations, but the fast response demonstrated in this study possibly indicates an

additional rapid effect on MCC. Improving disturbed MCC by enhanced CFTR activity and Cl^- secretion resulting in elevated fluid secretion might aid clearance of pathogens and debris in the inflamed airways.

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Author Contributions: Mandy Laube and Ulrich H. Thome conceived and designed the experiments; Miriam Bossmann, Benjamin W. Ackermann and Mandy Laube performed the experiments; Mandy Laube and Miriam Bossmann analyzed the data; Mandy Laube and Miriam Bossmann wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AKT	Protein kinase B
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
Dexa	Dexamethasone
GCs	Glucocorticoids
GR	Glucocorticoid receptor
I_{SC}	Short-circuit current
MCC	Mucociliary clearance
NDRG1	N-myc downregulated gene 1
NEDD4L	Neural precursor cell expressed, developmentally downregulated 4-like
mTORC2	Mammalian target of rapamycin complex 2
PDK1	Phosphoinositide-dependent kinase
PI3K	Phosphoinositide 3-kinase
R_{te}	Transepithelial resistance
SGK1	Serum and glucocorticoid dependent kinase 1
V_{te}	Transepithelial voltage

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6. Zusammenfassung

Dissertation zur Erlangung des akademischen Grades *doctor medicinae* (Dr. med.)

Der Einfluss von Glukokortikoiden auf die tracheale Chloridsekretion

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eingereicht am: Juli 2021

6.1 Hintergrund und Ziel:

Ionenbewegungen über das Atemwegsepithel bilden die Grundlage einer definierten Zusammensetzung intraluminaler pulmonaler Flüssigkeit. Ein perinataler Anstieg maternaler und damit einhergehend fetaler Glukokortikoid (GC)-Serumlevel ist vergesellschaftet mit einer Veränderung im epithelialen Ionentransport der Lunge. So erfährt das respiratorische Epithel in einer perinatalen Anpassungsreaktion eine Transition fetaler Fruchtwasserproduktion hin zu überwiegend flüssigkeitsabsorbierenden Vorgängen. In den distalen Abschnitten spiegelt sich dies in einer Aktivierung flüssigkeitsabsorptiv wirkender epithelialer Natriumkanäle (ENaC) wider. Vor Beginn der Transition überwiegt ein sekretorisch aktiver, apikaler Chloridkanal, der *cystic fibrosis transmembrane conductance regulator* (CFTR). Als Gegenspieler zum ENaC gewährleistet der CFTR einen transepithelialen Flüssigkeitsstrom in das Lumen des respiratorischen Systems. Diese Prozesse ermöglichen den pulmonalen Gastaustausch sowie die Bereitstellung eines periziliären Flüssigkeitsfilms, welcher die postnatale mukoziliäre Clearance (MCC) gewährleistet. Während bereits gezeigt werden konnte, dass GC den ENaC vor allem in distalen Atemwegsepithelien in Expression und Aktivität steigern (Thome et al. 2003; Venkatesh und Katzberg 1997), gibt es bisher nur wenige Daten zum Einfluss von GC auf den CFTR.

Das Ziel dieser Arbeit ist es, der Frage nachzugehen, welchen Einfluss GC auf die Aktivität des CFTR ausüben und welche Regulationskaskaden z.B. im Proteinkinasenetzwerk dem unterliegen. Dazu wurden die Unterschiede im Ionentransport wie auch in der RNA- bzw. Proteinexpression des CFTR unter Beeinflussung kinaseabhängiger Signalwege untersucht.

6.2 Ergebnisse

(die Ergebnisse beziehen sich, solange nicht anders erwähnt, auf das proximale respiratorische Epithel der Ratte)

1. GC haben keinen Einfluss auf die epitheliale Barrierefunktion (TEER).
2. GC steigern sowohl cAMP- als auch spezifisch CFTR-abhängige Ionenströme.
3. Die CFTR-mRNA-Expression wird durch GC reduziert.
4. Die Hemmung der Phosphoinositol-3-Kinase (PI3K) beeinflusst die basale CFTR-Aktivität nicht.
5. Die Hemmung der PI3K negiert jedoch die GC-induzierte Steigerung der CFTR-Aktivität.
6. Die PI3K-Inhibition beeinflusst basale sowie GC-induzierte cAMP-abhängige Ionenströme
7. Die SGK1-Aktivität wird durch GC gesteigert, was durch eine Hemmung von GC-Rezeptoren (GR), als auch durch eine direkte Inhibierung der SGK1 verhindert wird.
8. Eine Aktivierung sowie eine Hemmung der SGK1 beeinflusst jedoch die GC-vermittelte Erhöhung der CFTR-Aktivität nicht.
9. Eine Hemmung der PKB/AKT hemmt den basalen sowie den GC-stimulierten CFTR-abhängigen Ionentransport.
10. Die GC-induzierte Zunahme der AKT-Phosphorylierung und die damit einhergehende Steigerung der AKT-Aktivität wird sowohl durch eine GR-Blockade als auch durch eine AKT-Inhibition verhindert.
11. GC steigern die NEDD4L-Phosphorylierung und inhibieren dadurch die Ubiquitinligase.
12. Die GC-induzierte NEDD4L-Phosphorylierung wird durch eine GR-Blockade als auch durch eine AKT-Inhibition verhindert.
13. Eine Hemmung der SGK1 hat keinen Einfluss auf den GC-stimulierten Phosphorylierungslevel der NEDD4L.
14. GC erhöhen die CFTR-Aktivität in Calu-3 Zellen innerhalb von 30 Minuten.
15. Die mRNA-Expression des CFTR bleibt nach 30-minütiger Inkubation mit GC unverändert.
16. GC steigern die mRNA-Expression des TMEM16A/Ano 1.

Zusammenfassend kann durch die vorliegende Arbeit gezeigt werden, dass es durch GC zu einer Zunahme des CFTR-abhängigen Ionentransportes kommt und dass dieser aktivitätssteigernde Einfluss von der PI3K sowie der AKT abhängig ist, während die SGK1 nicht wesentlich in diesen Wirkmechanismus involviert zu sein scheint. Es ließ sich nachweisen, dass es durch GC zu einer gesteigerten Phosphorylierung von AKT, SGK1 sowie NEDD4L kommt, woraus eine Aktivitätssteigerung der AKT und der SGK1 sowie ein Rückgang in der NEDD4L-Aktivität resultieren. Eine AKT-Inhibition verhindert die zunehmende NEDD4L-Phosphorylierung, während eine Inhibition der SGK1 keinen Einfluss auf den NEDD4L-Phosphorylierungsstatus zeigt. Des Weiteren ergibt sich ein stimulierender Einfluss von GC auf die CFTR-Aktivität innerhalb von 30 Minuten. Im Gegensatz zum Ionentransport zeigten GC keinen Einfluss auf den Epithelwiderstand und damit die Barrierefunktion der Zellkultur.

7. Diskussion

Die Grundlage zur Untersuchung eines möglichen Einflusses von GC auf die Ionenkanalaktivität im proximalen Atemwegsepithel stellt eine primäre Zellkultur des respiratorischen Epithels der adulten Ratte dar. Dieses Tiermodell wurde aufgrund von histologischen Untersuchungen des Atemapparates unterschiedlicher Tiermodelle gewählt, welche gezeigt haben, dass das zumeist genutzte Mausmodell in den Zellverteilungsmustern wenig Ähnlichkeit zum Menschen besitzt. Die Histologie der Atemwegsepithelien der Ratte ähnelt viel mehr dem humanen Vorbild. (Widdicombe et al. 2001)

Zu Beginn meiner Arbeit erfolgte der Ausschluss einer GC-bedingten Änderung des basalen Epithelwiderstandes in der Zellkultur. Würde z.B. die Ausprägung interzellulärer tight junctions beeinflusst werden, wie es berichtet wurde (Sekiyama et al. 2012), hätte es durch Dexamethason zu einer Zunahme des Epithelwiderstandes, verglichen mit der Kontrollgruppe, kommen müssen. Daraus folgt, dass GC keinen Einfluss auf die Integrität und damit auf die transzelluläre Permeabilität des Zellverbandes proximaler respiratorischer Epithelien haben. Daher kann davon ausgegangen werden, dass die erhobenen Messungen am ehesten auf Veränderungen der Kanalaktivitäten basieren.

Die Ergebnisse hinsichtlich der hier untersuchten Ionenbewegungen über das respiratorische Epithel deuten eine Unterteilung des Atemapparates in zwei Kompartimente an. Das proximale Epithel des Bronchialbaumes sowie der Trachea steigert im Gegensatz zum alveolaren Epithel seine Chloridsekretion als Antwort auf GC. Dieser Unterschied in der Reaktivität spiegelt sich auch in der funktionellen Unterteilung in luftleitendes sowie gasaustauschendes Gewebe wider. Hinsichtlich der physiologischen Aufgaben des Atemwegsepithels erscheinen die Ergebnisse schlüssig. So wird das nach GC-Anstieg dominierend absorptive Verhalten des respiratorischen Epithels in distalen Atemwegen wie den Alveolen genutzt, um *peripartum* intraluminales Fruchtwasser zu absorbieren und somit den einsetzenden alveolären Gasaustausch zu unterstützen. (Bland 2001) Die proximalen luftleitenden Atemwege jedoch würden die ihnen zugehörige Aufgabe nicht erfüllen können, wenn auch dort parallel zum fetalen pränatalen GC-Anstieg der sezernierende Aspekt des Epithels verloren ginge. So könnte die Befeuchtung des inspiratorischen Luftstromes nicht mehr suffizient stattfinden. Es käme zu einer Störung in der Zusammensetzung der periziliären Flüssigkeitsschichten (PCL), mit der Folge einer eingeschränkten MCC mit insuffizientem Abtransport gelöster Pathogene und Partikel. (Pilewski und Frizzell 1999; Tarran et al. 2006) Denkbar wären daraus resultierende akute wie auch chronische Atemwegsinfektionen sowie chronische Umbaureaktionen des pulmonalen Gewebes aufgrund des anhaltenden inflammatorischen Reizes. (Livraghi und Randell 2007; Zhou-Suckow et al. 2017) Hinsichtlich einer iatrogenen Einschränkung der MCC durch erhöhte Viskosität der PCL aufgrund von Sauerstoffapplikation oder Beatmung ohne Befeuchtung gibt es widersprüchliche Ergebnisse. Franchini et al zeigten in ihrer Studie keinen signifikanten Einfluss einer Befeuchtung des Sauerstoffs bei einer Fallzahl von 18 Probanden sowie *low flow* Sauerstofftherapie, wohingegen andere Studien ergaben, dass sich bei angefeuchteter *high flow* Sauerstofftherapie oder invasiver Beatmung mit Befeuchtungseinrichtung, im Vergleich zur Kontrollgruppe, eine verbesserte MCC mit verbesserter Zilienfunktion einstellt. (Franchini et al. 2016; Chalon et al. 1972; Salah et al. 1988) Die Expression des CFTR-Gens, gemessen anhand der CFTR-mRNA-Menge, zeigt sich trotz der beschriebenen funktionellen Unterschiede in der CFTR-Aktivität jedoch weiterhin supprimiert durch GC, unabhängig von der Herkunft des Epithels, was einen Einfluss von GC auf posttranslationalem Level vermuten lässt. Bestätigend zeigten Prota et al., dass GC auf posttranslationalem Level durch Regulation der Chaperon-Interaktionen das CFTR-Protein Trafficking und damit die CFTR-Membranexpression erhöhen, während zeitgleich die CFTR-Gen-Expression reduziert wurde. (Prota et al. 2012)
In diesem Zusammenhang wurden Kinasen mit bekanntem Einfluss auf epitheliale Transportprozesse untersucht. Die Ergebnisse lassen vermuten, dass die PI3K eine bedeutende Rolle in der Regulation des

CFTR einnimmt. Hierbei scheint die PI3K, zumindest im proximalen respiratorischen Epithel, keine Rolle in der Regulation der basalen CFTR-Aktivität zu spielen. Dies steht im Widerspruch zum Rückgang der basalen CFTR-Aktivität in Calu-3 Zellen nach PI3K-Inhibition. Hieraus lässt sich vermuten, dass zelltypspezifische Regulationsschritte in die Aktivierungskaskade des CFTR eingebunden sind. So könnten noch weitere Kinasen wie z.B. die PIKfyve (Gehring et al. 2009), CK2 (Luz et al. 2011) oder auch P2Y2-Rezeptoren involviert sein. (Faria et al. 2009; Mendes et al. 2011) In der Literatur wird über weitere Kinasen *downstream* der PI3K mit Einfluss auf epitheliale Ionenflüsse berichtet.

Die SGK1 jedoch, durch GC in ihrer Aktivität gesteigert (Lang und Stournaras 2013), beeinflusst die CFTR-abhängigen Cl-Ströme im proximalen Epithel der Atemwege nicht. So war die GC-induzierte Aktivitätszunahme wie auch die erhöhte basale CFTR-Aktivität nach SGK1-Hemmung unverändert nachweisbar. Die Wirkung des verwendeten SGK1-Inhibitors wurde mittels NRDG1-Phosphorylierungs-Assay bestätigt. Durch eine Hemmung des GC-Rezeptors (GR) konnte die SGK1 ebenso inhibiert werden. GC stimulieren daher zwar die SGK1, jedoch ohne eine daraus resultierende Veränderung der CFTR-Aktivität. Im Gegensatz zu diesem Ergebnis zeigen Arbeiten an *Xenopus* Oozyten, dass durch Coexpression der SGK1 die CFTR-Aktivität gesteigert werden konnte. (Wagner et al. 2001; Sato et al. 2007) Caohuy et al (Caohuy et al. 2009) bestätigen in CFPAC-1 Zellen, einer Zelllinie aus dem Adenokarzinom des Pankreasgangepithels eines CF Patienten (deltaF508), dass GC die CFTR-Protein- und Oberflächenexpression sowie die Kanal-Halbwertszeit steigern, eine Inhibition der SGK1, des GR oder der PI3K diesen Effekt jedoch negieren. Übereinstimmend damit scheint eine GC-vermittelte Steigerung der SGK1 eine gesteigerte deltaF508- sowie Wildtyp-CFTR-Membranexpression in CFBE41o- Zellen zu induzieren, indem der endozytotische Abbau inhibiert wird. (Rubenstein et al. 2011; Bomberger et al. 2014) In den dargestellten Messungen zeigte die SGK1 keinen Einfluss auf die CFTR-Aktivität. Jedoch wurde in unserer Arbeit auch nicht die Oberflächenexpression oder das CFTR-Trafficking, sondern die Kanalaktivität bestimmt. Die Menge eines Kanals ist dabei nicht gleichzusetzen mit der daraus resultierenden Offenwahrscheinlichkeit bzw. der Ionenkanalaktivität. Andere Studien, die sich mit den gegensätzlichen Ergebnissen aus SGK1-Aktivitätsmessungen auseinandergesetzt haben, vermuten, dass zelltypspezifische Ausstattungen mit z.B. *Anchoring*-Proteinen eine Ursache darstellen könnten. (Wilson et al. 2010; Snyder et al. 2002)

Im Gegensatz zur SGK1 ist die AKT unverzichtbar für die Aktivität des CFTR sowie für die aktivitätssteigernde Wirkung der GC. Es konnte nicht nur gezeigt werden, dass der GC-stimulierte CFTR-Ionenstrom sowie die basale CFTR-Aktivität durch AKT-Inhibition stark reduziert werden, sondern auch, dass GC zu einer Zunahme der Ser473-Phosphorylierung führen und dadurch zu einer Aktivitätszunahme der AKT. Eben diese Phosphorylierung lässt sich durch den Akt-Inhibitor AKT1/2 blockieren. Dementsprechend wirkt sich auch eine GR-Hemmung mit Mifepristone reduzierend auf den Phosphorylierungsstatus der AKT aus. Eine potentiell unspezifische Wirkung des AKT1/2 auf die SGK1 (Mansley und Wilson 2010) kann vernachlässigt werden, da eine SGK1-Inhibition selbst keinen Einfluss auf der CFTR-Aktivität zeigte.

NEDD4L, ein weiterer Kandidat im regulatorischen Netzwerk um den CFTR, führt durch Ubiquitinylierung von Membranproteinen zu deren Abbau. (Staub et al. 1997; Li et al. 2007; Goulet et al. 1998) In Calu-3 Zellen konnte gezeigt werden, dass GC zu einer gesteigerten Phosphorylierung der NEDD4L und damit zur Reduzierung der Aktivität im Ubiquitinylierungsprozess führen. Durch die Phosphorylierung kommt es zur Anlagerung des regulatorischen Proteins 14-3-3 an die NEDD4L, woraufhin die Bildung eines für den Abbau von Proteinen essentiellen Enzym-Substrat-Komplexes verhindert wird. (Nagaki et al. 2006) Übereinkommend mit den Ussing Kammer Messungen, verhindert die GR-Blockade den Effekt von GC auf die NEDD4L-Phosphorylierung. Hinsichtlich des ENaC konnte bereits gezeigt werden, dass eine NEDD4L-Phosphorylierung zu einer Zunahme der ENaC-Oberflächenexpression führt, indem der endozytotische Abbau reduziert wird. (Snyder et al. 2002;

Debonneville et al. 2001) Über den Zusammenhang von NEDD4L und CFTR gibt es unterschiedliche Angaben. In CF-Pankreaszellen nimmt die Expression von deltaF508 nach NEDD4L-Knockdown zu, (Caohuy et al. 2009) wohingegen weder Überexpression noch Knockdown der NEDD4L in *Xenopus* Oozyten bzw. in CFBEwt-Zellen zu einer Beeinflussung der wtCFTR-Oberflächenexpression oder der Chloridströme führt. (Koeppen et al. 2012) Die Autoren dieser Veröffentlichungen vermuten, dass NEDD4L über positive wie auch negative Regulatoren einen indirekten Einfluss auf die CFTR-Oberflächenexpression und Aktivität ausübt. (Koeppen et al. 2012) Genauer berichten die Arbeiten von einem Anstieg in der CFTR-Kanaldichte, jedoch ohne parallelen Anstieg der CFTR-Aktivität, während nach NEDD4L-knockdown die CFTR-Ströme ohne Zunahme der Kanaldichte ansteigen. So scheint sich die Inhibierung der NEDD4L nur im Netto-Ionentransport auszuwirken. Letztendlich erhärtet sich die zuvor aufgestellte Hypothese, dass die Menge an exprimierten CFTR an der Zelloberfläche nicht direkt in Zusammenhang zur messbaren Aktivität des CFTR gebracht werden kann.

Erwähnenswert ist auch die Tatsache, dass sowohl die AKT, als auch die SGK1 bekannt dafür sind, NEDD4L durch Phosphorylierung zu inhibieren. (Snyder et al. 2002; Debonneville et al. 2001; Lee et al. 2007) Eine Inhibierung der AKT führt dementsprechend auch zu einer Abnahme der CFTR-Aktivität, übereinstimmend mit der Hypothese, dass es nach einer AKT-Inhibition zu einem vermehrten CFTR-Abbau kommt. Andererseits wenn die SGK1 die NEDD4L in ihrer Aktivität beeinflussen kann, wäre zu erwarten, dass eine SGK1-Inhibition die CFTR-Aktivität beeinflussen würde. Unsere Ergebnisse stützen daher einer PI3K/AKT-abhängige Stimulation der CFTR-Aktivität durch GC, welche möglicherweise über Inhibition der NEDD4L wirkt. Eine direkte Interaktion zwischen CFTR, NEDD4L bzw. NEDD4L und AKT konnte in dieser Arbeit nicht nachgewiesen werden und bedarf weiterer Experimente.

Abschließend konnten ein signifikanter Anstieg der CFTR-Aktivität in der Ussing Kammer innerhalb von 30 Minuten nach GC-Applikation nachgewiesen werden. Interessanterweise konnte eine gesteigerte Aktivität der AKT in A549 Zellen schon nach zwei Minuten nachgewiesen werden, welche durch eine Inhibierung der PI3K verhindert wurde. (Kayahara et al. 2005) Es konnte auch ein Anstieg der Phosphorylierung der AKT, der NEDD4L sowie NDRG1 innerhalb von 30 Minuten nach GC-Applikation gezeigt werden, wohingegen die CFTR mRNA -Expression unverändert blieb. In der Zusammenschau sehe ich die Hypothese gestärkt, dass es sich beim GC-vermittelten Anstieg der CFTR-Aktivität um eine Regulation auf nicht-genomischer Ebene handelt. Innerhalb einer Zeitspanne von ≤ 30 Minuten von Applikation bis zum Wirkbeginn der GC sollte nicht von genomischen Effekten ausgegangen werden, da für die Regulation der nukleärer Expressionseffekte nach bisherigem Kenntnisstand eine gewisse Latenzzeit notwendig ist. (Falkenstein et al. 2000; Kino et al. 2011) So sieht Urbach et al einen Zusammenhang in der Hemmung der schnellen GC-Wirkung durch Inhibierung der Adenylatzyklase. (Urbach et al. 2006; Urbach et al. 2002) Ferner wird vermutet, dass GC über membranständige G-Protein gekoppelte GC-Rezeptoren mit der Adenylatzyklase interagieren. Ähnliches ist für die ebenfalls steroidalen Östrogene bekannt, für die membranständige Östrogen-Rezeptoren als GPCR am endoplasmatischen Retikulum beschrieben wurden. (Tasker et al. 2006)

Zusammenfassend eröffnet diese Arbeit die Möglichkeit zum weitergehenden Verständnis der physiologischen Regelkreise hinsichtlich der Einhaltung einer Flüssigkeitshomöostase sowie der Umstellung eines Gleichgewichtes auf neue Aufgaben im respiratorischen Epithel. Ein wachsendes Verständnis über den Einfluss von GC auf die tracheale Chloridsekretion und MCC könnte hinsichtlich therapeutischer Fragestellungen bei chronisch entzündlichen Atemwegserkrankungen aufgrund des häufig damit einhergehenden Einsatzes von GC in der Akut- wie auch in der Erhaltungstherapie an Bedeutung gewinnen. So konnte letztendlich gezeigt werden, dass GC einen kinaseabhängigen stimulierenden Einfluss auf die CFTR-Aktivität und somit auf die Chloridsekretion im luftleitenden respiratorischen Epithel besitzen.

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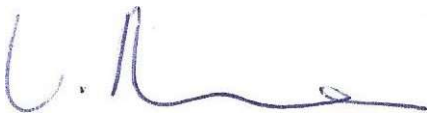
9. Anmerkungen zur Originalpublikation – Darstellung des eigenen Beitrags

Zur Publikation unter dem Titel "Glucocorticoids Distinctively Modulate the CFTR Channel With Possible Implications in Lung Development and Transition into Extrauterine Life" ist anzumerken, dass der von mir eigenständig geleistete Beitrag die primäre Zellkultur des Atemwegsepithels betrifft. Hierzu zählen die Etablierung der Zellgewinnung sowie Kultivierung als auch die Durchführung der elektrophysiologischen Messungen und Analysen der mRNA Expression. Des Weiteren wurde an der Erstellung der Publikation mitgewirkt.

Die Etablierung der Zellkultivierung sowie die Durchführung als auch die Auswertung der Experimente und die Niederschrift zur Publikation "Signaling Cascade involved in Rapid Stimulation of Cystic Fibrosis Conductance Regulator (CFTR) by Dexamethasone" basiert in hauptsächlichem Maße auf meiner eigenständigen Arbeit.



Prof. U. Thome

Dr. M. Laube

Dr. B. Ackermann

10. Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

Lübeck, Juni 2021

11. Curriculum vitae

entfernt

12. Danksagung

Nun ist es an der Reihe „Danke“ zu sagen. So sehr ich diesen Moment der Vervollständigung meiner Promotionsschrift in all den Jahren mühevoller Arbeit herbeigesehnt habe, so herausfordernd ist es jetzt, meinen empfundenen Dank all den hieran Beteiligten im Geschriebenen gerecht zu werden.

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Auch wenn mein Name auf dem Deckblatt der Dissertationsschrift zu finden ist, so steht dieser nur stellvertretend für all die Menschen, ohne die diese Arbeit nicht zu verwirklichen gewesen wäre. All jedem gebührt mein aller herzlichster Dank!