Modulating adipogenesis via signaling pathways and cellular redox state

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vorgelegt von

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Contents

Nomenclature

AD	adenylyl cyclase
ADD-1	adipocyte determination and differentiation factor-1
bcl-2	B-cell lymphoma-2
bFGF	basic fibroblast growth factor
8-Br-cAMP	8-bromoadenosine-3',5'-cyclic monophosphate
8-Br-cGMP	8-bromoguanosine-3',5'-cyclic monophosphate
cAMP	cyclic adenosine monophosphate
C/EBPα-δ	CAAT enhancer binding protein alpha-delta
cGMP	cyclic guanosine monophosphate
cGK	cGMP-dependent protein kinase
cGMP-PDE	cyclic guanosine monophospate-phosphodiesterase
CHOP-10	C/EBP homologous protein-10
CREB	cyclic adenosine monophospate-response element-binding protein
CRP	C-reactive protein
csGC	cytosolic solulable guanylyl cyclase
DDA	2',5'-dideoxyadenosine
DETA	dietylentriamine
DMEM	Dulbecco's modified Eagle medium
EGCG	epigallocatechin gallate
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
FCS	fetal calf serum
FK614	3-(2,4-dichlorobenzyl)-2-methyl-*N*-(pentylsulfonyl)-3-*H*benzimidazole-5-carboxamide
FKHR	forkhead transcription factor
GPDH	glycerophosphate dehydrogenase
GSK3β	glycogen synthase kinase 3 beta
HIF-2α	hypoxia-inducible factor 2 alpha
HO-1	heme oxygenase-1

IBMX	isobutylmethylxanthine
IGF-1	insulin-like growth factor-1
INF-γ	interferon gamma
iNOS	inducible nitric oxide synthase
LiCl	lithium chloride
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
Mn-SOD	manganese superoxide dismutase
NF-κB	nuclear transcription faktor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
ODQ	1H-(1',2',4')oxadiazolo-(4',3'-a)quinoxalin-1-one
PBS	phosphate buffered saline
PCR	polymerase chain reaction
8-pCPT-cGMP .	8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate
PDE-II	cGMP-induced cAMP-phosphodiesterase
PDE-III	cGMP-inhibitied cAMP-phosphodiesterase
PIP	phosphatidylinositol 3-kinase-protein kinase B
PKC	protein kinase C
PKA	protein kinase A
PLGA	polylacticcoglycolic acid
PPARγ	peroxisome proliferators-activated receptor-gamma
PRKG	protein kinase G
ROS	reactive oxygen species
RT	reverse transcription
RXRα	retinoic acid receptor-alpha
SREBP-1c	sterol regulatory element-binding protein-1c
TGF-β	transforming growth factor beta
TNFα	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

1 Introduction

1.1 Research on adipogenesis: historical background

Adipogenic precursor cells, so called preadipocytes, have been receiving increasing attention in the context of obesity, type-2-diabetes and other uprising diseases of the well-nourished western hemisphere [1]. In order to understand fat neo-formation, energy homeostasis, and adipose tissue physiology, preadipocytes have become an attractive tool for researchers. The first immortal cell line of preadipocytes was created by Green and colleagues [2] in the early 1970s. Around 1980, fat cells were mainly seen as a depot for the storage of energy while the process of storing or releasing lipids was managed by hormones [3–5]. The notion that indeed fat cells themselves are actively interfering with the energy homeostasis of the human body emerged over the last decade: novel molecular approaches discovered signaling molecules such as TNF-α, leptin, or plasminogen activator-1 to be produced by adipocytes and highly influencing fat metabolism in an autocrine and paracrine manner [6–11]. One of the most important approaches of this decade was the demonstration of the multipotent character of human preadipocytes: after adequate hormonal activation, they are able to differentiate into osteoblasts, endothelial cells, myoblasts, chondrocytes and several other cell types [12–17]. However, the most natural branch of differentiation in preadipocytes remains the adipogenic one.
Another pivotal aspect of research on adipose tissue has been pursued in this decade: the reconstruction of subcutanous fat tissue in patients with soft tissue defects. These defects may result from post-traumatic defects after extensive burns, tumor defects, or congenital deformities [18]. Thus, there is an urgent need to find new approaches and techniques to restore the missing tissue because of its protecting and modeling attributes representing a mechanical cushion for muscles, tendons, and bones [18].

1.2 Preadipocytes: a tool for adipose tissue engineering

1.2.1 Problems and limits of soft tissue engineering

Techniques of reconstructing soft tissue defects have been established in plastic surgery over the last few decades and involve local and free flaps, dermal fat grafts, collagen injections, the use of synthetic materials, and free adipose tissue grafts [18,19]. Disadvantages such as foreign body reactions appear especially in methods using synthetic materials whereas biologically derived transplants often shrink to an unpredictable extent [20]. Also, fat injection has not yet presented satisfying results [21]. Unfortunately, free adipose tissue grafts are almost completely absorbed and replaced by fibrous tissue and oil cysts [22, 23]. This is due to an insufficient vascularization and blood supply, causing central hypoxia and cell necrosis [24]. A more promising way of reconstructing soft tissue defects could be the embedding of autologous progenitor fat cells in biological scaffolds. This method has become a well-studied field of research in the last years [19, 25, 26].

1.2.2 Application of preadipocytes for soft tissue engineering

The exact definition of preadipocytes remains unclear: recent studies analyzed surface antigenes in order to find an appropriate marker for the cell group of adipogenic precursors. These cells belong to the so called stromal-vascular fraction of adipose tissue [27]. They are able to differentiate into a variety of cells including endothelial cells, osteoblasts, myoblasts, chondrocytes, and fat cells [12, 13, 17]. Unfortunately, a specific marker for adipose progenitor cells has not been found yet which outlines the difficulty of exact characterization. Since this is a broad field of study, my thesis will refer to adipogenic precursors as preadipocytes. The focus of this thesis was the differentiation process of fibroblast-like adipose progenitors to lipid-filled mature adipocytes suggesting that the main componend of isolated cells is able to convert into adipocytes and can therefore be called "preadipocytes".

Preadipocytes are considered a reservoir for new fat formation since in contrast to mature fat cells, they have the ability to proliferate and differentiate into adipocytes [28] after appropriate stimulation. Their putative role in soft tissue engineering can be explained by their good ability to divide and be grown in cell culture as well as their good resistance against nutrient and oxygen shortcome compared to mature adipocytes [29]. In 1999, Patrick et al. introduced a polylactic-coglycolic acid (PLGA) polymer disk as a matrix for preadipocytes. Since then, various scaffolds and non-wovens with varying materials, porosity, and size have been created and examined [18, 19, 26, 30–32]. In 2005, Hemmrich

et al. presented an *in vivo* model of human preadipocytes seeded on hyaluronic-acid based scaffolds which were implanted into immunodeficient mice. Their results were encouraging since human preadipocytes were able to survive transplantation and even showed extensive differentiation at the recipient site and vascularization of the surface [33]. However, there is still an urgent need to improve differentiation of preadipocytes especially in larger implants *in vivo*. An insufficient differentiation causes cell necrosis and fibrotic tissue. A transplanted preadipocyte-loaded scaffold with these attributes will not meet the requirements for modeling and refilling extensive soft tissue defects. *In vitro* studies on optimizing preadipocyte culture and differentiation are therefore the basis for further *in vivo* experiments on preadipocyte-loaded scaffolds.

1.3 Mechanisms in adipogenesis

1.3.1 Preadipocyte proliferation and differentiation

In order to optimize preadipocyte culture and differentiation, the underlying physiological processes need to be well understood. The two main steps involved in adipogenesis are proliferation and differentiation of progenitor fat cells. Differentiation is the transition from undifferentiated fibroblast-like preadipocytes into mature round lipid-filled fat cells [34] (Fig. 1.1). Proliferating preadipocytes undergo growth arrest initiated through contact inhibition. Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through the subsequent differentiation steps, leading to the progressive acquisition of the morphological and biochemical characteristics of mature adipocytes [35]. This model postulates that proliferation of preadipocytes occurs prior to differentiation. Indeed many studies using cell lines such as 3T3-L1 preadipocytes [36, 37] came to this conclusion, whereas studies using human preadipocytes indicate that partially differentiated cells remain capable of replication [38]. In their fully differentiated state, adipocytes are incapable of cell division although there have been some reports of the replication of mature adipocytes *in vitro* [39, 40].

1.3.2 Signaling cascades

In vitro studies show that preadipocyte proliferation and differentiation are defined by intrinsic and extrinsic factors including adipose depot, donor age, and androgen or estrogen status [41–43]. In cell culture, hormonal stimulation of the differentiation process has been extensively investigated [28, 40, 44, 45]. Differentiation of confluent preadipocytes

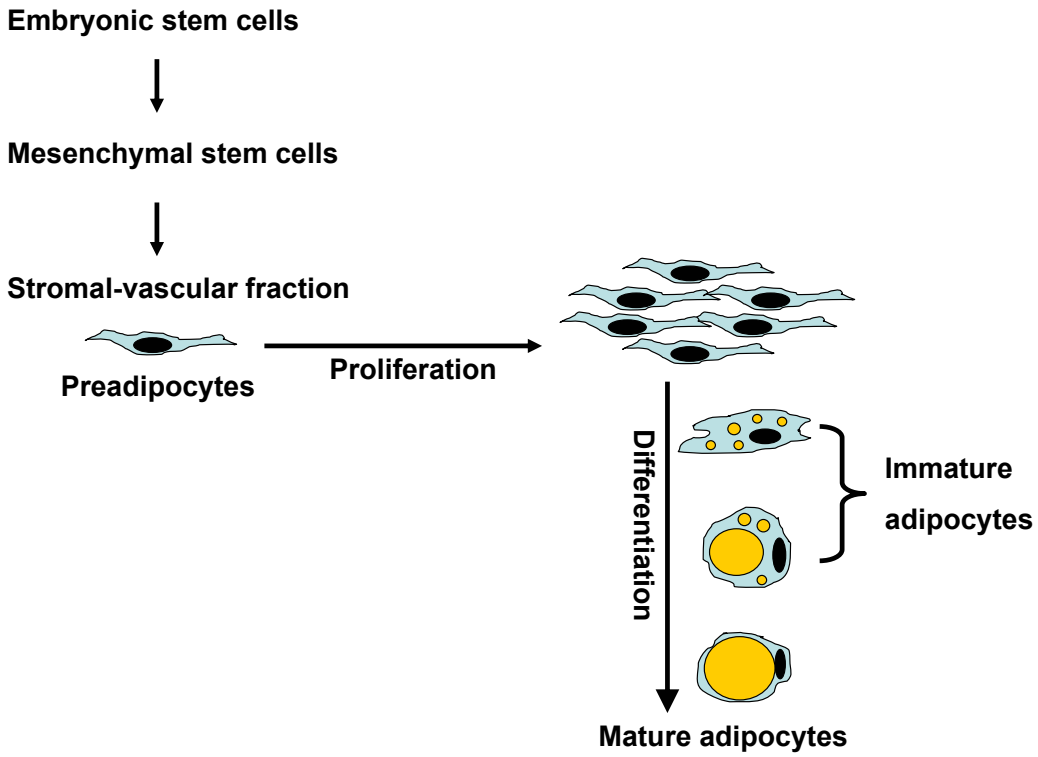

Figure 1.1: Schematic representation of the major processes of adipogenesis. Deriving from mesenchymal stem cells, preadipocytes represent a cell group of the stromal-vascular fraction in the adipose tissue. After hormonal stimulation, preadipocytes proliferate (*in vitro* until confluency) before the process of differentiation can be induced. During differentiation, the cells collect lipid vacuoles until they become all lipid-filled mature adipocytes.

is induced by hormones such as insulin, but also by glucocorticoids, phosphodiesterase inhibitors, and others [44,45]. Fig. 1.2 - Fig. 1.4 illustrate the complex intracellular mechanisms. Transmembrane proteins such as insulin-like growth factor receptor (IGFR)-1 and members of the epidermal growth factor (EGF)-like protein family respond to extracellular stimuli. These trigger intracellular cascades which induce or repress transcription factors that modulate adipogenesis. Novel studies ascertain two kinase systems that are activated by IGF-1 receptor signaling: MAP kinases and phosphatidylinositol 3-phosphate protein-kinase B/Akt [46–48]. The latter inhibits the forkhead transcription factor Foxo 1 (FKHR) which was recently discovered to lower the activity of PPARγ, one of the key regulators in adipogenesis [48–52]. Lipophilic substances such as glucocorticoids, insulin sensitizers, and others directly interact with cytosolic receptors thereby influencing gene

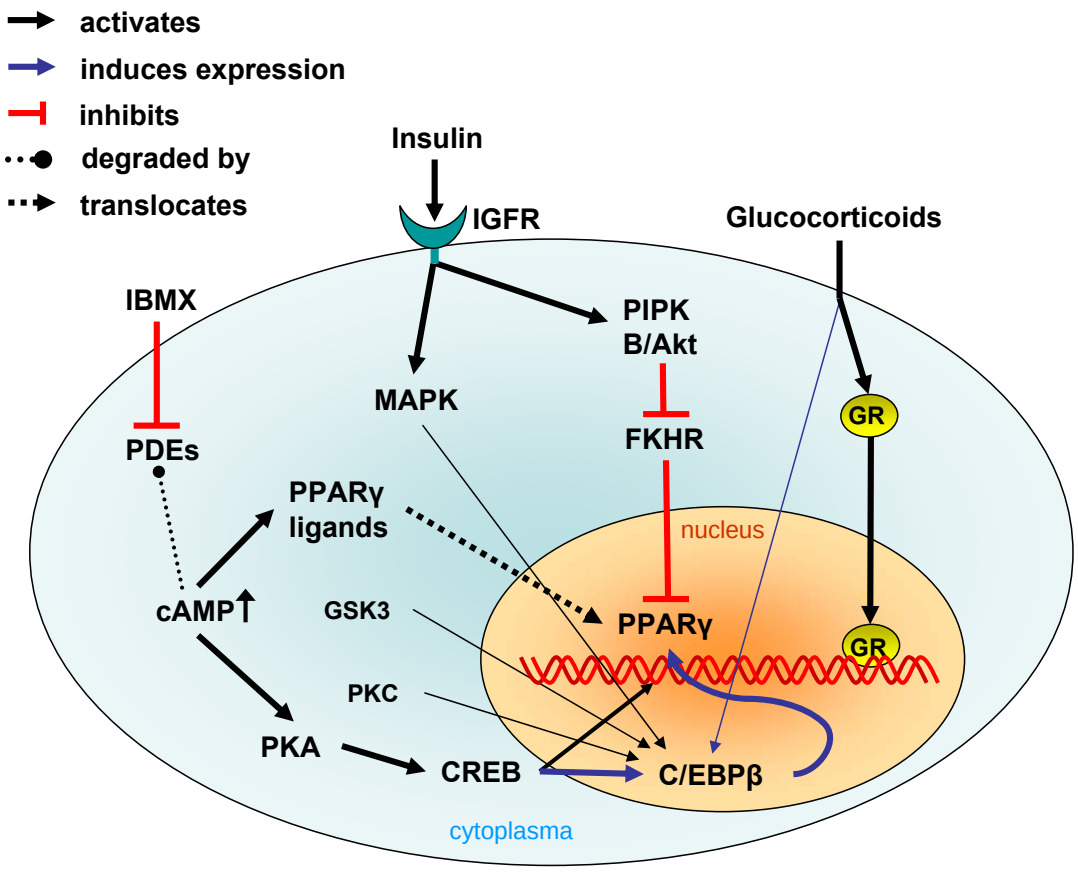

Figure 1.2: Hormonal induction of adipogenesis. Intracellular cascades which influence pro-adipogenic transcription factors are triggered by metabolic regulators such as insulin, dexamethasone, and IBMX. Insulin-induced activation of the IGF-receptor induces pathways involving MAP kinases and PIP-kinase B/Akt. Glucocorticoids directly induce C/EBPβ and bind to an intracellular receptor which activates other transcription factors. IBMX promotes differentiation via unspecific inhibition of phosphodiesterases which leads to higher cAMP levels. cAMP acts through protein kinase A targeting the cAMP element binding protein (CREB). CREB induces expression of C/EBPβ (also activated by GSK3β, PKC, and MAPK) which stimulates adipogenesis via induction of PPARγ.

transcription (compare Fig. 1.2). Apart from direct interaction with their intracellular receptor which targets specific DNA-sequences, glucocorticoids have recently been shown to induce C/EBPβ, a transcription factor at an early state of adipogenesis [53]. C/EBPβ is one of the key regulators of adipogenesis and is also induced by an intracellular cascade which is set off by isobutylmethylxanthine (IBMX) [28]: the IBMX-induced inhibition of phosphodiesterases (PDEs) elevates adenylyl monophosphate (cAMP)-levels which even-

tually target specific binding proteins (CREBs) [28]. CREBs can also be activated by PKC, MAPK, and GSK3β [54–56]. The DNA-binding capacity of CREB is most likely activated by hyperphosphorylation [57]. At the end of the cascade, C/EBPβ and C/EBPδ induce the expression of PPARγ which in turn activates C/EBPα [28]. These two proteins are the central transcriptional regulators of adipogenesis targeting adipocyte-specific genes [58]. The protein ADD-1/SREBP-1c accelerates adipocyte differentiation, regulating the expression of PPARγ, and providing ligands for this receptor [59, 60] (Fig. 1.3). PPARγ as well as ADD-1/SREBP-1c and C/EBPα are therefore useful adipogenic markers for PCR or western blot analysis.

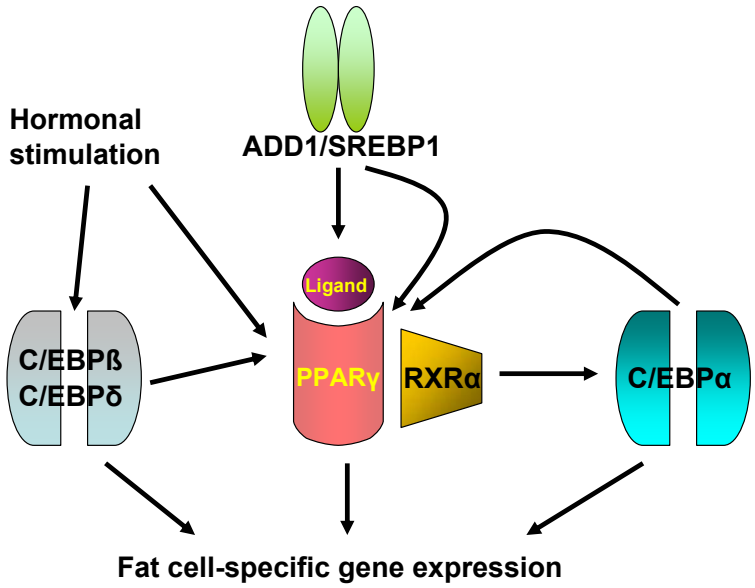

Figure 1.3: Major transcription factors involved in preadipocyte differentiation. Hormonal induction of transcription factors promotes the differentiation process. Early transcription factors, C/EBPβ and C/EBPδ, induce PPARγ which (in complex with RXRα) activates C/EBPα, thereby maintaining differentiation by feedback mechanisms. Another transcription factor, ADD-1/SREBP-1c, activates PPARγ by producing an endogenous ligand and by inducing its expression [figure modified after Rosen and Spiegelman 2000].

The complex network of adipogenesis can be influenced at many sites where extra- or intracellular stimuli interfere with the cascades described above. One example can be given by the family of heat shock proteins (HSP): HSPs generally serve as intracellular chaperones, thereby controlling protein folding [61]. However, recent literature found evidence that they also interact with adipogenic processes: HSP70 and HSP90 were demonstrated to build complexes with glucocorticoid receptors and the peroxisome proliferators-activated receptor prior to ligand activation [61–63] (Fig. 1.4).

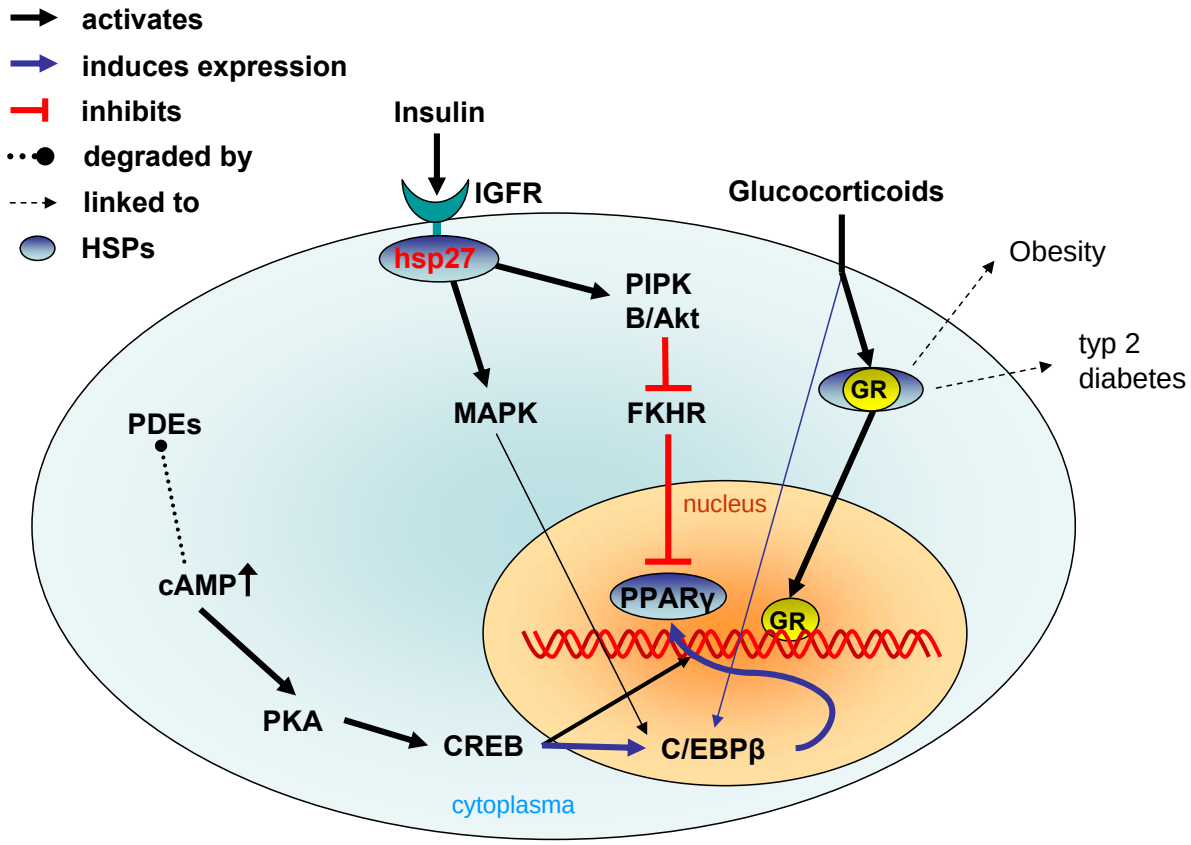

Figure 1.4: Role of heat shock proteins in adipogenesis. HSP70 and HSP90 build complexes with glucocorticoid receptors and PPARγ. Polymorphisms of both HSPs are linked to an increased risk for obesity and type 2 diabetes. HSP27 influences insulin sensitivity by chaperoning IGF-1 and Akt.

The relevance of this can be concluded from clinical studies which have linked polymorphisms in HSP70 to an increased risk for obesity and type 2 diabetes [64,65]. Furthermore, HSP27 interacts with the insulin-like growth factor receptor 1 and its signal transducer,

the serine/threonine kinase protein Akt, thereby influencing insulin sensitivity [66, 67] (Fig. 1.4).

1.3.3 Mitochondrial reactive oxygen species formation

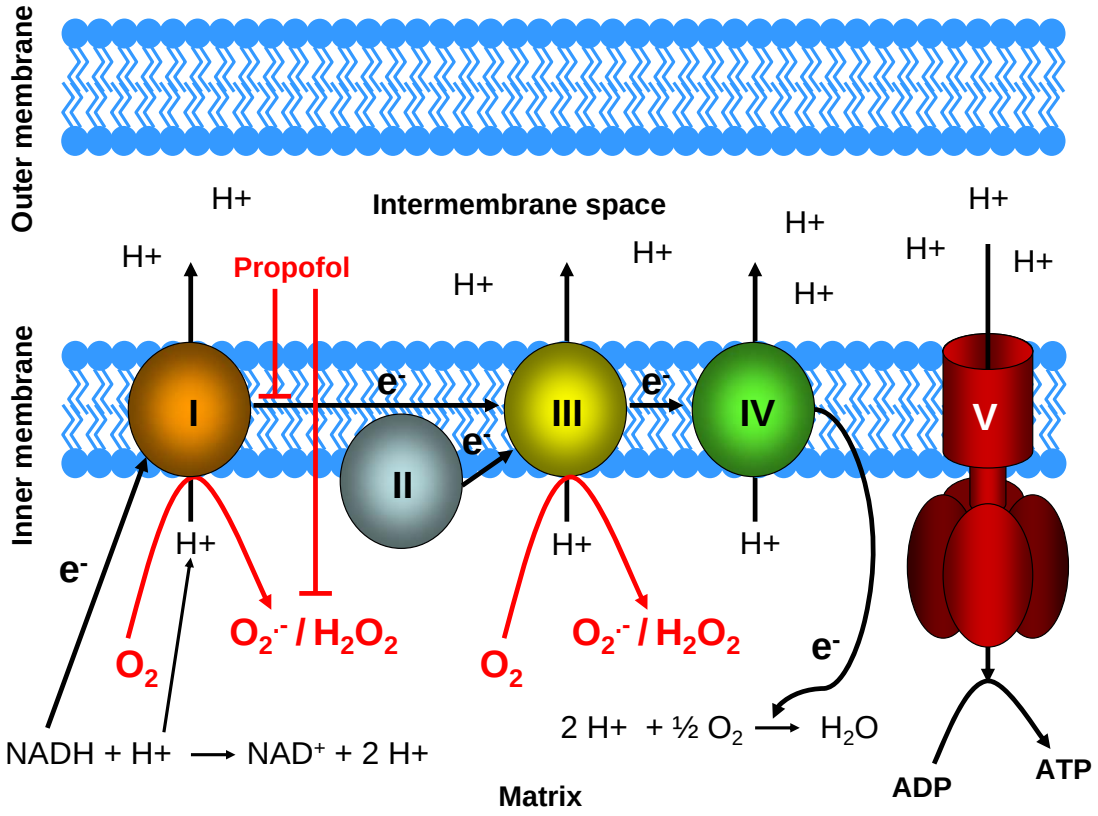

Figure 1.5: Mitochondrial ROS generation. The mitochondrial electron transport chain consists of different complexes (I-V). NADH carries protons (H+) and electrons (e-) to the electron transport chain located in the inner membrane. The energy from the transfer of electrons along the chain transports protons across the membrane and creates an electrochemical gradient. As the accumulating protons follow the electrochemical gradient back across the membrane through complex V (ATP synthase), the movement of the protons provides energy for synthesizing ATP. At the end of the electron transport system, two protons, two electrons, and half of an oxygen molecule combine to form water. A small percentage of the O_2 consumed by this transport chain is incompletely metabolized and reduced to superoxide anions ($O_2^{\cdot -}$) which are subsequently diverted into other ROS such as H_2O_2 by complex I and III. Propofol inhibits complex I and also scavenges free radicals at this site.

Besides appropriate hormonal stimulation [68], the cellular redox status regulates the balance between proliferation and differentiation in most cell types [69]. Even cell-extrinsic signaling molecules like growth factors have been demonstrated to induce extensive progenitor self-renewal by changing the intracellular redox state. In general, free radicals can cause severe damage to biological systems: increased levels of oxidative stress are associated with inhibition of tumor growth, blockade of cell cycle progression and induction of apoptosis [70,71]. The concept of mitochondrial reactive oxygen species (ROS) generation is as follows: mitochondria, which have been recognized to be involved in adipose tissue plasticity [72], constitute the primary source of ROS generation in many cells. The outer mitochondrial membrane contains complexes of integral membrane proteins that form channels through which a variety of molecules and ions can pass. The inner membrane contains 5 complexes of integral membrane proteins (Fig. 1.5): NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase (Complex III; also known as the cytochrome b-c1 complex), cytochrome c oxidase (Complex IV), and ATP synthase (Complex V). In cellular respiration, energy is obtained through the transfer of electrons down the electron transport chain. Electrons are removed from an electron donor (NADH or $FAHD_2$) and passed down the electron transport chain to a terminal electron acceptor (O_2) via a series of redox reactions (Fig. 1.5). Complexes I, III, and IV use this energy to pump protons from the mitochondrial matrix into the intermembrane space. The resulting transmembrane proton gradient is used to make ATP via ATP synthase. As can be seen from Fig. 1.5, a small percentage of the consumed O_2 is, however, incompletely metabolized and reduced to a superoxide anion radical ($O_2^{\cdot -}$) by complex I and complex III according to their steady state reduction [73]. Propofol is able to inhibit complex I and scavenge free radicals at this site [74] (see Fig. 1.5). Superoxide anions are diverted into secondary products such as hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD).

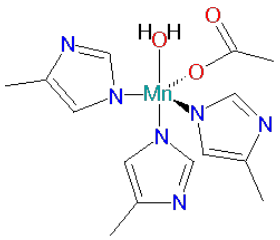

Figure 1.6: Schematic representation of manganese superoxide dismutase of human mitochondria

Superoxide dismutases were first discovered in 1969 by McCord and Fridovich [75] and prevent the intracellular accumulation of radicals. In mitochondria, these enzymes have a manganese ion (Mn) in their active center (Fig. 1.6) whereas cytosolic and intracellular variants hold copper/zinc (Cu/Zn) centers. The reduced form of the metal (MnII or CuI)

transfers superoxide anions to H_2O_2 (equation 1.1), followed by the oxidation of a second equivalent of $O_2^{\cdot-}$ through MnIII or CuII to O_2 (equation 1.2) [76]. The less aggressive H_2O_2 is degraded by other enzymes such as catalases and glutathione peroxidase.

$$O_2^{\cdot-} + M^{n+} + 2H^+ \longrightarrow H_2O_2 + M^{(n+1)+} \tag{1.1}$$

$$O_2^{\cdot-} + M^{(n+1)+} \longrightarrow O_2 + M^{n+} \tag{1.2}$$

Net reaction:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \tag{1.3}$$

An endogenous overproduction of superoxide anions is linked to a reduced ability to eliminate free radicals (= oxidative stress) and can cause severe damage to biological systems [77]. Thus, superoxide dismutases are already commercially available at pharmacies praised to have anti-inflammatory and cell protecting abilities [76].

1.3.4 Oxidative stress and adipogenesis

In adipogenesis, free radical formation has been demonstrated to inhibit fat cell formation by reducing C/EBP binding capacity [78]. In particular, mitochondrial ROS are substantially involved in adipogenic processes: ROS are linked to redox-sensitive signal transduction activation such as MAPK and Akt that promotes the phosphorylation of HIF-1α [79, 80] (Fig. 1.7). Phosphorylation increases the transcriptional activity of HIF-1α via the dimerization with HIF-1β [81]. The heterodimer represses PPARγ promoter activation [82] (Fig. 1.7) and activates the expression of transcription factors depending on the cell type [81]. A second model for the anti-adipogenic effects of ROS was introduced by Carriere [74, 83] and Galinier [84]: they found that hypoxia partially inhibits mitochondrial electron transport, producing redox changes in the electron carriers that increase ROS generation [74]. Hypoxia-dependent inhibition of adipocyte differentiation was associated with an increase of HIF-1α, as expected, but they also found an independent mechanism of ROS controlled expression of CHOP-1, a negative regulator of C/EBPs, which forms heterodimers with these proteins (Fig. 1.7) [85]. These observations were supported by Galinier et al. [84] in experiments with Zucker rats: the adipose tissues of obese individuals showed a specific higher content of hydrophilic molecules in a lower redox state than those of lean rats indicating that obesity is associated with reduced oxidative stress.

ROS are also involved in insulin resistance of mature adipocytes by down-regulating GLUT-4 expression [86]. The effects of HIF transcription factors on adipogenesis may, however, be different under normoxia conditions: Shimba at al. recently demonstrated that members of the C/EBP family are also able to induce HIF-2α [87, 88]. Under normoxia, HIF-2α then acted as a promoter of adipogenesis by direct transcriptional activation of glucose transporters such as GLUT1 and GLUT4 [87, 88] (Fig. 1.7). This leads to improved cell metabolism and therefore favours preadipocyte differentiation.

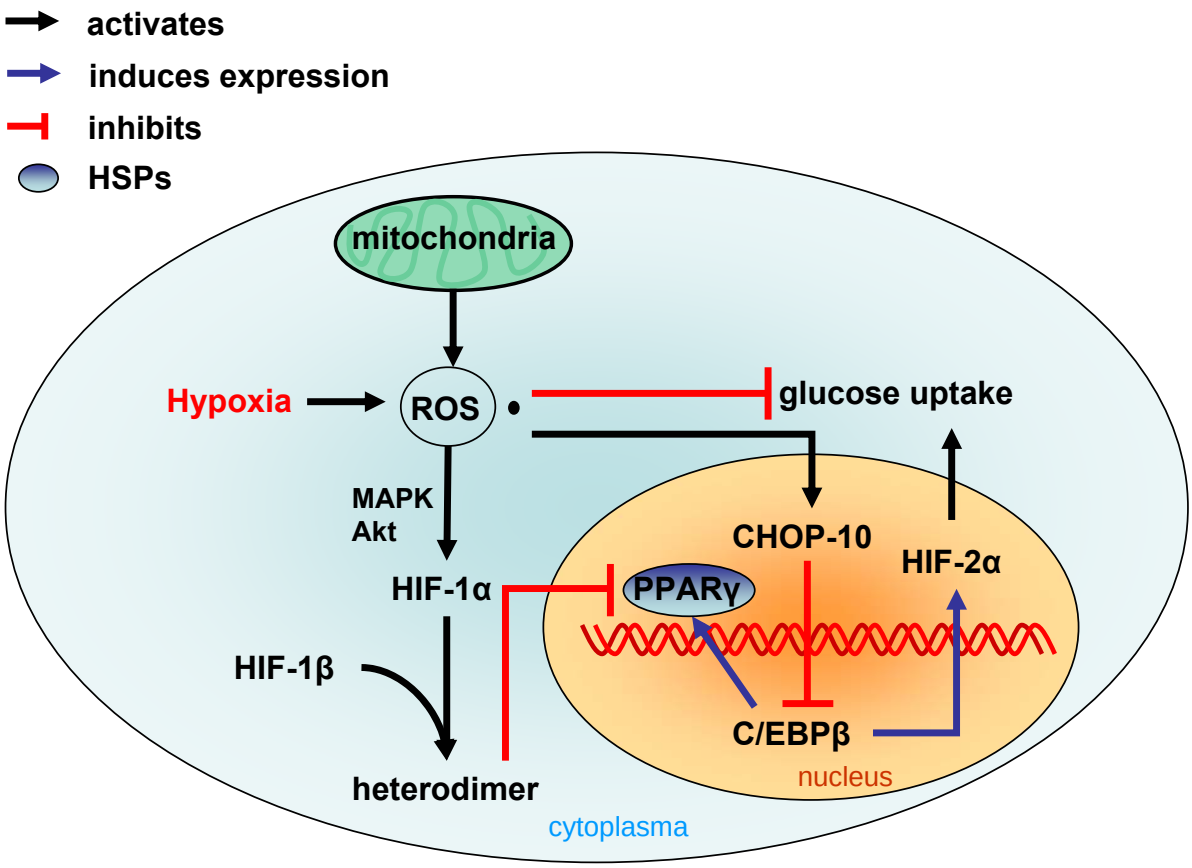

Figure 1.7: Intracellular modification of signaling pathways by reactive oxygen species and hypoxia. Mitochondrial ROS highly influence adipogenesis, especially under hypoxic conditions, via induction of CHOP-10, a negative regulator of PPARγ, down-regulation of certain glucose transporters, and promotion of heterodimerization of HIF-1. The subsequent transcriptional acitivity leads to repression of PPARγ promoter activation. Under normoxia, C/EBPß induces HIF-2α, thereby enhancing cellular insulin sensitivity.

In conclusion, the reduction of oxidative stress does not only promote the differentiation process from preadipocytes to mature fat cells, it may also affect insulin sensitivity and energy homeostasis in general [78]. Besides their potential to influence adipogenesis, ROS are involved in the development of degenerative diseases and aging [89]. They can function as physiological signaling molecules [90] and second messengers in the O_2-sensing mechanism triggering gene transcription of erythropoietin and vascular endothelium growth factor [91]. Various cytoplasmic enzymes such as HO-1 or SOD-1 respond to increased ROS levels which emphasizes the specific gene-regulating properties of ROS inside the cell.

1.4 Effects of nitric oxide

1.4.1 Intra- and extracellular effects of nitric oxide in the human body

The signal molecule nitric oxide (NO) is a central mediator of multiple physiological and pathophysiological functions throughout the body. NO is synthesized by the enzyme nitric oxide synthase (NOS) from L-arginine and in the presence of molecular oxygen resulting in equimolar concentrations of NO and citrulline. NO has a half-life of seconds and is usually oxidized to the inactive but stable endproducts nitrite and nitrate (NO_2 and NO_3^-) [92]. In this study, the donor-molecule DETA (dietylentriamin) that releases NO for 24 hours was used to provide a continuous concentration of NO in the culture medium.
There are several isoforms of the NOS enzyme that have been isolated and studied: two of them are constitutively expressed NO synthases: endothelial and neuronal NOS (eNOS and nNOS). Endothelial derived NO is best known for its function as a regulator of the vascular tone [93]. Neuronal NO has important functions in the central nervous system such as formation of memory [94]. Furthermore, NO can act as a neurotransmitter causing relaxation in the gastrointestinal tract and the corpus cavernosum mediating penile erection which makes the combination of sildenafil - a competitive binding agent of cGMP specific phosphodiesterase type 5 in the corpus cavernosum - and NO-donors so dangerous [95–98]. Additionally, after activation by proinflammatory cytokines (e.g. interleukin-1ß, tumor necrosis factor-α, interferon-γ, etc.) and/or bacterial products (e.g. lipopolysaccharides (LPS)), an inducible NO synthase (iNOS) is expressed producing NO for prolonged periods of time (hours to days) in an apparently unregulated fashion [99, 100]. Intracellular NO-mediated signaling pathways are classified as either being

cyclic guanylic acid (cGMP)-dependent or -independent. cGMP formation results from NO binding to the heme group of soluble guanylyl cyclase, thus leading to enzyme activation. The cGMP-independent mechanisms include inhibition of iron-sulfur centers, DNA damage, polyadenosine 5'-diphosphate-ribosylation activation with cellular energy deprivation, protein modification due to S-nitrosation of thiol groups, and, in the context of concurrent oxidative stress, tyrosine nitration, which causes the antimicrobial, cytostatic or cytotoxic effects of NO [101–104]. Yet, the picture of how NO influences signal transduction remains complex and is far from being completely understood.

1.4.2 Nitric oxide and adipogenesis

With the identification of eNOS and iNOS in adipose tissue [105, 106], increasing recognition has emerged for a regulating role of NO in fat cell formation: in 2002, Yan et al. discovered an enhancement of differentiation in rat white adipose tissue [107]. Hemmrich et al. found that NO inhibits preadipocyte proliferation while promoting adipogenic conversion to mature fat cells *in vitro* [108]. *In vivo* experiments linked NO to increased insulin sensitivity of the white adipose tissue and inhibition of lipolysis in subcutaneous adipose tissue depots [109–114]. In 2004, Engeli et al. found increased iNOS expression in adipogenic differentiation and proposed NO to be an important mediator in adipocyte physiology with lipogenic abilities [115]. Since iNOS is a key effector enzyme of inflammation (see section 1.4.1), it drives NO production after activation and therefore reinforces and maintains the inflammatory response [116]. Further, an increasing body of evidence has emerged that inflammatory processes are linked to the formation of adipose tissue: a state of chronic low-level inflammation with raised plasma levels of inflammatory cytokines and acute-phase proteins is found in obesity and the metabolic syndrome [117–121]. Increased plasma levels of cytokines and acute-phase proteins lead in turn to an up-regulation of iNOS gene expression [115] which promotes further adipose tissue formation by enhancing lipid storage and by decreasing basal and catecholamine-induced lipolysis [109–114, 122, 123].

1.4.3 Possible modulation of preadipocyte differentiation in soft tissue engineering

Preadipocytes are potential targets of eNOS- or iNOS-synthesized NO. Effects of NO on preadipocytes are rarely characterized to date [107]. The direct link between NO and adipogenic precursor cells is the unavoidable inflammatory response which will ap-

pear in every setting of implanted cell-loaded scaffolds for reconstruction purposes. As mentioned above, a present focus of attention in tissue engineering is the insufficient maturation of preadipocytes, especially in the center of larger three-dimensional constructs. The reason for this is not yet completely understood. Although insufficient blood and oxygen supply are suspected to be the main reasons, inflammation might be a so far underestimated factor. In order to analyze effects of NO on preadipocytes in a setting as found after transplantation of a three-dimensional cell-seeded biohybrid, primary human preadipocytes were treated with DETA/NO and NO-mediated effects on proliferation and adipogenic conversion was investigated. Possible intracellular mechanisms are described in Fig. 1.8. Besides NO, modulators of adenylyl and guanylyl cyclases - two enzymes which play a major role in the differentiation process - and cAMP and cGMP-analogues were examined for their potential to enhance preadipocyte differentiation (see Fig. 1.8). I looked at the specific ability of each substance to modulate the differentiation process and combined activators of both cyclases with NO and cAMP/cGMP-analogues. Specific inhibitors of both enzymes (ODQ = guanylyl cyclase inhibitor and DDA = adenylyl cyclase inhibitor) were added separately and combined in order to elucidate the NO-effect on guanylyl cyclase as well as adenylyl cyclase. In a second step I tried to modify the standard medium (DMEM/Ham's F12 + IBMX, pioglitazone, transferrin, dexamethasone, insulin, and triiodo-L-thyronine) by omitting certain reagents and adding guanylyl/adenylyl cyclase activators and cGMP/cAMP-analogues. I therefore tried to facilitate and enhance the differentiation procedure of preadipocytes which lasts about 14 days under standard medium conditions. A modified culture medium and a thorough understanding of the intracellular mechanisms could be useful in tissue engineering in terms of facilitating preadipocyte differentiation in three-dimensional carriers *in vitro* before implantation or in already implanted transplants by hormonal stimulation *in vivo*.

1.5 Pharmaceutical modulation of adipogenesis

1.5.1 Clozapine-induced weight gain: clinical relevance and possible mechanisms

If adipogenesis can be influenced by various hormones and transmitters, the question arises of whether certain drugs that cause weight gain in patients - such as atypical neuroleptics, lithium chloride, or antidepressants - can have a direct influence on preadipocyte maturation as well. With atypical neuroleptics, the exact mechanisms of increased weight

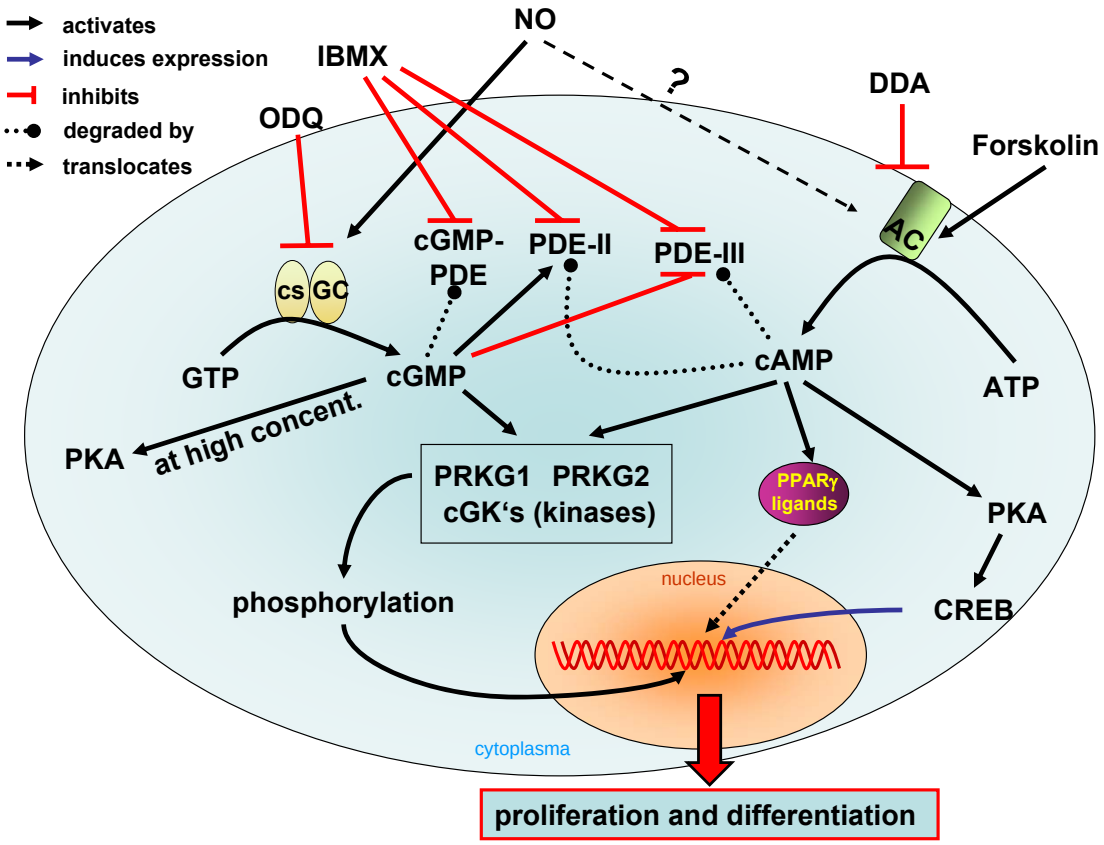

Figure 1.8: Intracellular effects of nitric oxide, activators, and inhibitors of adenylyl cyclase and guanylyl cyclase. As in other cells, nitric oxide may act through guanylyl cyclase in preadipocytes, too. However, also adenylyl cyclase activation has to be considered as a potent intracellular signaling cascade. ODQ inhibits cytosolic solulable guanylyl cyclase (csGC). Forskolin is an activator of membrane bound adenylyl cyclase (AC); 2'-5'-Dideoxyadenosine (DDA) inhibits this enzyme. IBMX unspecifically inhibits cGMP-phosphodiesterase (cGMP-PDE), cGMP-induced cAMP-phosphodiesterase (PDE-II), and cGMP-inhibited cAMP-phosphodiesterase (PDE-III) thereby elevating intracellular cGMP and cAMP levels [124]. Both cGMP and cAMP activate protein kinase G 1 and 2 (PRKG1, PRKG2) and cGMP-dependent protein kinase (cGK) which then phosphorylate transcription factors promoting adipogenesis. cAMP also activates specific PPARγ ligands (see Fig. 1.2 and Fig. 1.3).

in patients is not yet fully understood. Among the atypical neuroleptics, olanzapine and clozapine show the greatest weight-increasing potential [125, 126]. This weight gain stabilizes in the short to medium term but often continues beyond the first year, leading to weight increases of up to 20-30 kg [127]. The treatment-emergent weight gain has

been beneficial in observing and anticipating the therapeutic effect of neuroleptics in patients [128]. However, adverse health effects such as cardiovascular diseases and insulin resistance as well as psychological problems bear a risk for the physical health of the patient. These side-effects may lead to frustration and non-compliance and therefore justify an intensive examination of the mechanisms responsible for the neuroleptics-induced weight gain. One common hypothesis for the pro-adipogenic effect of clozapine involves the hormone leptin, which is produced mainly in white adipose tissue and plays a major role in the regulation of energy homeostasis and food intake by targeting the leptin receptor in the hypothalamus [129]. Supporting this hypothesis, clozapine has been found to increase serum leptin levels within days after starting treatment [130]. Thus, the perception of satiety is impaired and patients experience a lower suppression of appetite after meals [131]. Other studies focussed on plasma levels of insulin, adiponectin, ghrelin and even nitric oxide [132–135]. Unfortunately, these studies present controversial results concerning plasma levels of such hormones involved in energy homeostasis. To extend the understanding of the pathophysiology of clozapine-induced weight gain, the question of whether preadipocytes are a target for clozapine has to be thoroughly analyzed. It might lead to the insight that the increase in weight is a neo-formation of adipose tissue from precursor cells.

1.5.2 Effects of clozapine, lithium chloride and propofol on adipogenesis

Pro-adipogenic side effects in pharmaceuticals often cause adverse health effects and have a negative influence on the patient's compliance (see section 1.5). Unfortunately, the exact mechanisms of how these drugs interfere with lipid regulatory mechanisms are poorly understood. As in atypical neuroleptics, the pro-adipogenic effects, especially of clozapine and olanzapine, are controversially discussed in the literature [129,130,132–135]. However, a detailled analysis of intracellular clozapine-effects in preadipocyte maturation has not been given yet. Imaginable is an induction of adipogenic genes by intracellular cascades as observed for IBMX, insulin, or glucocorticoids (see section 1.3.2). Indeed, some studies describe the involvement of guanylyl cyclase (as in nitric oxide-mediated cascades) and dopamine-receptor mediated activation of adenylyl cyclase in the context of clozapine in neurons and other cell types [136–138]. The effects of clozapine inside the cell could also be explained by the contrary mechanism of superoxide production and peroxide scavenging, as recently demonstrated for radical scavengers such as propofol [74, 83]. Propofol, a commonly used sedative and anesthetic agent, is chemically similar to the endogenous

antioxidant α-tocopheral (vitamin E) [139], and has demonstrated antioxidant functions at organ and cellular levels: it efficiently attenuates oxidant injury in myocardial tissue, mitochondria, brain synaptosomes, and erythrocyte membranes [140–143]. That is why propofol is especially useful for anesthesia in patients with critical illnesses who are undergoing surgery that abnormally enhances oxidative stress [144, 145]. Its anti-oxidant and free radical-scavenging abilities lead to reduced generation of mitochondrial reactive oxygen species [74]. As mentioned above, every change in the sensitive balance of the intracellular redox sytem can cause severe alterations of gene transcription and set off intracellular cascades. Carriere et al. postulated that the propofol-induced reduction of ROS-generation causes increased preadipocyte differentiation [74, 83]. This led us to the assumption that the clozapine-effect might be due to similar mechanisms which became the basis for our study design.

1.5.3 Green tea: a potential remedy?

Green tea is a widely-consumed beverage in Asia and has been regarded to have health-promoting effects for centuries [146]. During the last decade, the traditional notion that green tea consumption causes beneficial health effects has received scientific attention and became the focus of intensive research [147–150]. Green tea and its main active components, the catechins, of which EGCG is the most abundant [148, 151], is said to have beneficial health effects due to its antioxidant activity leading to protection of organs systems, tissues, and cells from damaging free radicals [152]. EGCG has demonstrated anti-atherogenic and anti-coagulatory effects in various contexts [147, 153, 154]. Studies show that the polyphenols in green tea reduce the risk of cancer at several sites by stimulating the production of immune system cells [155]. Interestingly, EGCG is also known for its weight-reducing abilities which are due to metabolic and intracellular effects: there are reports on EGCG-mediated glucose uptake inhibtion by competitive inhibition of sodium/glucose cotransporters [156], reduced α-amylase and sucrase activities [157], and inhibition of gastrointestinal enzymes involved in nutrient digestion [146]. EGCG is also known to reduce levels of adipogenesis-related transcriptional factors, such as C/EBPß and PPARγ [158], and to suppress the activation of enzymes responsible for lipid metabolism, e.g. sterol regulatory element binding protein-1, in adipose tissue [159]. Various studies show that EGCG reduces size and number of lipid droplets and GPDH-activity in differentiating preadipocytes [160]. This effect can be explained by the above mentioned reduction of adipogenic transcriptional factors. Since EGCG has been found to liberate H_2O_2 [161], its effect on cytotoxity and especially tumor growth has been extensivly

studied: the free radical H_2O_2 inhibits growth of tumor cells by oxidative stress, blocking cell cycle progression, inducing apoptosis or causing necrosis in a dose and cell-type dependent manner [70]. Evidently, the susceptibility to EGCG is cell type specific and depends on the cell's ability to maintain its redox balance [71]. Normal human keratinocytes, for example, are capable of reducing oxidative stress and are therefore less susceptible to damage [162]. Referring to these findings and trying to find a link between anti-adipogenic effects of EGCG in preadipocyts and hydrogen peroxide production, I looked at the effects of EGCG on manganese superoxide dismutase which catalyzes the reaction from superoxide anions to hydrogen peroxide. Moreover, Chan et al. recently discovered, that EGCG can inhibit the growth of ovarian cancer cell by amplifying the cytotoxic effect of cisplatin which is most likely due to enhanced intracellular H_2O_2 generation [162]. Thus, hydrogen peroxide generation represents another mechanism for the anti-adipogenic effects of EGCG. Summarizing these observations, cellular redox status, Mn-SOD activity, and hydrogen peroxide generation could explain intracellular effects of EGCG as well as of clozapine. EGCG most likely promotes Mn-SOD expression thereby influencing adipogenesis. Clozapine, however, could have an inhibitory effect on Mn-SOD expression (see Fig 1.9). Supposing that EGCG might counteract the clozapine-induced neo-formation of fat cells, drinking green tea could present an easy but effective way to decrease neuroleptics-induced weight gain in patients.

1.6 Aim of this study

Examining and modulating preadipocyte proliferation and differentiation can be helpful for adipose tissue engineering as well as for understanding and treating clozapine-induced weight gain: in the first study, the role of NO in regulating proliferation and differentiation of human preadipocytes was analyzed. To elucidate the intracellular mechanisms of NO on preadipocyte differentiation, inhibitors and activators of guanylyl and adenylyl cyclase and cGMP/cAMP-analogues were added to the culture media. The differentiation medium was modified by omitting and adding certain reagents to find the most effective combination with the least reagents in order to facilitate future differentiation procedures. The results of this study are potentially helpful to modify present tissue engineering applications in order to improve cell maturation and organization in three-dimensional biohybrids.

The second study focussed on the effects of the neuroleptic clozapine on preadipocyte proliferation and differentiation in order to find a new explanation for weight gain in patients

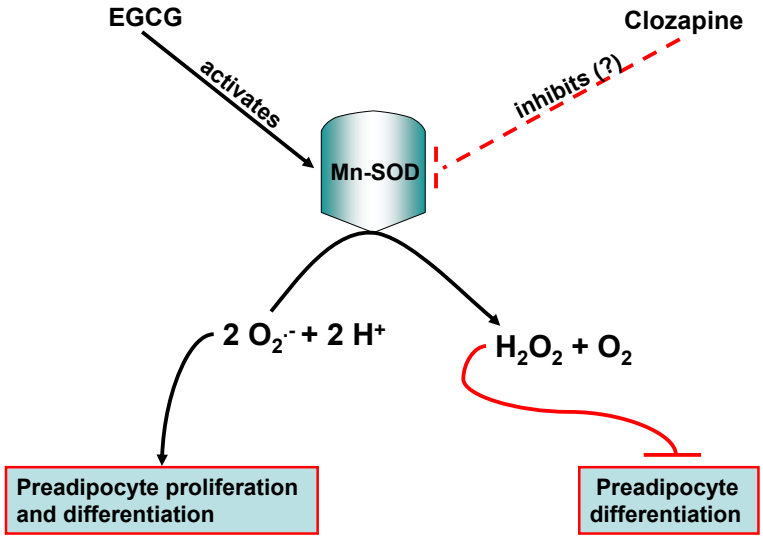

Figure 1.9: Modulation of Mn-SOD activity by EGCG and clozapine. EGCG activates Mn-SOD activity leading to higher intracellular peroxide-levels which inhibit differentiation. Clozapine may act through inhibition of Mn-SOD thereby causing higher superoxide anions levels thereby influencing preadipocyte proliferation and differentiation.

under long-term clozapine-treatment. Morphological changes and intracellular effects of clozapine on preadipocytes were investigated. Furthermore, the particular combination of clozapine and green tea was tested in order to determine its potential to decrease preadipocyte differentiation.

1.7 Study design

Preadipocytes were grown in standard culture medium (DMEM/Ham's F12) *in vitro* after being isolated from human adipose tissue samples. Proliferation and differentiation was evaluated separately and induced by common growth factors and hormones. Proliferating and differentiating cells were treated with NO at varying concentrations applied in form of a slow release carrier (DETA) which also served as a control to exclude any adipogenic effects. Proliferation was assessed by cell counting and evaluation of absorption changes due to mitochondrial conversion of a formazan dye (XTT-assay). Differentiation was measured via evaluation of enzyme activity of adipogenic conversion (GPDH-assay) and photo

documentation. Our hypothesis was based on studies about rat preadipocytes in which differentiation was highly promoted under NO addition [107]. We expected a rather inhibiting effect of NO on preadipocyte proliferation according to their cell physiology which favours either the proliferating or the differentiating branch. Intracellular effects of guanylyl and adenylyl cyclase-dependent pathways were evaluated by inhibitors and promoters of both enzymes as well as analogues of cAMP and cGMP. All conditions were also treated with DETA/NO to reveal possible intracellular mechanisms and their potential role in NO signaling. Assumed were strong participation of cGMP-dependent pathways and minor effects of cAMP-dependent mechanisms according to the literature [163–165]. Culture media were subsequently modified with cGMP and cAMP-analogues to facilitate and enhance culture conditions for preadipocyte differentiation.

The effect of clozapine on proliferating and differentiating cells was evaluated in a second study. Propofol and LiCL served as controls. Propofol was previously described to have a promoting effect on preadipocyte differentiation due to the reduction of ROS-generation by Carriere et al. [74]. LiCl has pro-adipogenic effects which are rather due to general metabolic activity such as increased glycogen synthesis [166] and glucocorticoid elevation [167], thus having no direct influence on preadipocyte differentiation. Since I assumed a promoting effect of clozapine on preadipocyte differentiation following similar patterns as described for propofol [74, 83], EGCG was added to clozapine-treated conditions in order to assess its potential ability to counteract the clozapine-effect by increased hydrogen peroxide production. This effect was hypothesized to be caused by enhanced Mn-SOD expression since EGCG has been reported to increase the activity of MnSOD in human hepatoma HepG2 cells and murine large intestinal cancerous epithelium [168,169]. Proliferation and differentiation were again evaluated by the above mentioned assays (XTT-assay, GPDH-assay), cell counting, photo documentation, and Oil-Red-O-staining of fat vacuoles in differentiated cells. PCR-analysis of superoxide dismutases was performed to evaluate activation of the MnSOD gene in EGCG-treated preadipocytes.

2 Materials and methods

2.1 Reagents

DETA was purchased from Aldrich (Taufenkirchen, Germany), collagenase solution type I 0.2%, PBS, and fetal calf serum (FCS) were from Biochrom (Berlin, Germany), triiodo-L-thyronine, transferrin, dexamethasone, basic fibroblast growth factor (bFGF), isobutylmethylxanthine (IBMX), 8-Br-cGMP, 2,5-dideoxyadenosine, 8-Br-cAMP, forskolin, propofol, hydroethidine, propidium iodide, and EGCG were from Sigma (Deisenhofen, Germany), 8-pCPT-cGMP was from Biolog (Bremen, Germany), ODQ from Alexis (Grünberg, Germany), pioglitazone from Takeda Pharmaceuticals (Lincolnshire, USA), Dulbecco´s modified Eagle medium (DMEM), trypsin and penicillin/streptomycin from PAA Laboratories (Colbe, Germany), lithium chloride (LiCl) from Merck (Darmstadt, Germany), and clozapine from Novartis (Nuremberg, Germany). The 250 μm-nylon sieve was from Verseidag Techfab GmbH (Geldern, Germany). DETA/NO was synthesized and isolated as described [170]. The GPDH-assay kit was from Takara (Otsu, Japan), the XTT-Kit from Roche (Mannheim, Germany), and the RNA-Isolation-Kit, the RNase-free DNase Set, the Omniscript-Kit, and the Taq core-Kit from Qiagen (Hilden, Germany).

2.2 Equipment

The "HeraCell240" incubator from Heraeus was used for cell incubation. For sterile working conditions all reagents were added under the laminar flow "Uniflow UVUB1800" from Uniequip. Microscopy was performed with a Zeiss microscope at 10x magnification. Water bath and shaking bath were from Gesellschaft für Labortechnik mbH (GFL), the vacuume system "Vacusafe" from Integra Bioscience. Reagents were weighed with a Sartorius precision scale and mixed with a magnetic stirrer from Heidolph. Centrifugation was performed with a "MinifugeT" centrifuge from Kendro and a "2-16KC" centrifuge from Sigma. Cell culture equipment such as pasteur pipettes was sterilized in the autoclave

"Autoklav 2540" from Systec. Changes of absorption in assays were measured by the ELISA-reader "MRX" from Dynex Technologies.

2.3 Cell isolation and culture conditions

For cell culture we used 96-, 12-, and 6-well-plates from Falcon and 25 cm^2 or 75 cm^2 culture dishes from Greiner Bio-One. Pipettes (25ml and 10ml) were from Costar whereas the 5ml-pipette was purchased from Techno Plastic Products (TPP). Pasteur pipettes were from Brand, pipette tips from Eppendorf. Tubes were from Falcon and beakers from VWR.

Preadipocytes were isolated from freshly excised human subcutaneous adipose tissue of patients (age: 18-67 years) who underwent elective operations (e.g. abdominoplasty) at the Department of Plastic Surgery and Hand Surgery - Burn Center of the University Hospital Aachen. Fibrous tissue and visible blood vessels were removed, the adipose tissue was minced and digested by collagenase solution type I (0.2%) at 37° C for 45 min with constant shaking. The ratio of tissue to enzyme was 1:1. After filtration (250 μm), the fat layer was removed and the cell suspension centrifuged at 720 G for 7 minutes. After resuspension of the pelleted cells in DMEM/Ham's F12 supplemented with 10% FCS, 0.1% bFGF, and 1% penicillin/streptomycin, cells were seeded onto 25 cm^2 or 75 cm^2 culture dishes and cultured at 37° C at 5% CO_2. The next day, cells were washed three times with NaCl before adding new medium. Afterwards, culture medium was changed every 3-4 days including two washing steps with PBS.

Growth, expansion, and differentiation of preadipocytes were carried out as follows: cells were grown to confluency in DMEM/F12 (1:1) supplemented with 10% FCS plus bFGF (10 ng/ml) and 1% penicillin/streptomycin. After two washing steps with PBS, cells were trypsinized for about 5 minutes. Medium (supplemented with serum) was added to stop trypsin activity. The cell suspension was centrifuged at 350 G for 10 minutes; the pellet was resuspended into medium. Cells were equally distributed onto 6-wells (for differentiating cells: ∼900.000 cells/well) or 12-wells (for proliferating cells: ∼60.000 cells/well). Adipogenic conversion was then promoted for 8-14 d by changing medium to DMEM/F12 (1:1) without serum addition, supplemented with 66 nM insulin, 100 nM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), 0.1 μg/ml pioglitazone, 1 nM triiodo-L-thyronine, and 10 μg/ml human transferrin (standard differentiation medium). After 5 days of incubation, medium was used as before but without IBMX. To evaluate the influence of NO on proliferation and differentiation, DETA/NO was added 6 days at 50 -

150 μM for proliferating and 5 days at 30 - 100 μM for differentiating cells. DETA/NO was activated by addition of 500 μl PBS and 0.3 μl HCl (1 N) per 0.82 mg DETA/NO. As a control, DETA was used at 150 μM or 100 μM, respectively. For identifying the underlying molecular pathway the specific guanylyl cyclase inhibitor ODQ (15 μM), the cGMP-analogue 8-Br-cGMP (1 mM), and the phosphodiesterase-stable cGMP-analogue 8-pCPT-cGMP (1 mM) were applied. Involvement of adenylyl cyclase was analyzed by addition of the specific adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (DDA) (25 μM), the cAMP-analogue 8-Br-cAMP (1 mM) and the adenylyl cyclase activator forskolin (10 μM). All reagents (except DETA/NO which had to be renewed on a daily base) were added every second day following a change of culture medium to avoid accumulation and toxicity. To evaluate the differentiation potential of preadipocytes, various differentiation-inducing factors were added to the differentiation medium (compare Table 2.1). To evaluate its influence on proliferation and differentiation, clozapine was added every second day at concentration levels between 5 μM and 50 μM. Propofol (10 μM), LiCl (2 mM), and EGCG (10 μM) were added every other day. The addition of all substances was stopped after 5 days in differentiation medium.

Table 2.1: Variation of culture media. Medium 1 represents the standard differentiation medium, media 2-6 vary in reagents but not in concentrations (same as in 1, see 2.3). Penicillin/streptomycin was added to each one of the media at a concentration of 1%.

	1	2	3	4	5	6
IBMX	x		x	x	x	
Pioglitazone	x	x				
Transferrin	x	x				
Dexamethasone	x	x	x	x		x
Insulin	x	x	x		x	x
Triiodo-L-thyronine	x	x				

2.4 Determination of proliferation

To determine the total number of viable preadipocytes, cells were detached from the dishes by trypsin treatment for 2-5 minutes, stained with trypan blue, and counted under the microscope in a Neubauer-chamber. To verify cell counting results, an XTT cell proliferation assay [171] was applied. The use of the tetrazolium salt XTT (2,3-Bis(2-methoxy-4-

nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) allows to analyze cell proliferation, cell viability, and/or cytotoxicity. Cleavage of the tetrazolium salt to formazan occurs via the succinatetetrazolium reductase system in the mitochondria of metabolically active cells. During this process, XTT, a yellow tetrazolium salt, is cleaved to a soluble orange formazan dye (see Fig. 2.1). Formazan formation in the supernatant was analyzed by

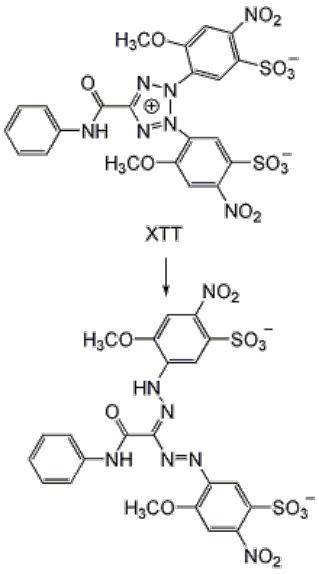

Figure 2.1: Formation of formazan dye. Cleavage of tetrazolium salts by enzymes of cells which are metabolically active lead to the formation of formazan crystals. Formazan formation in the supernatant of proliferating cells changes absorption wave length and gives rise to the amount of vital cells.

the ELISA-reader after treatment with 5 mg/ml XTT and incubation for 60 minutes at 37° C.

To determine the number of attached but necrotic cells, the trypan blue exclusion assay was applied: 9 g NaCl and 5 g trypan blue were added to 100 ml aqua dest. to get a 5% trypan blue solution. PBS (0.36 ml) was added to 0.27 ml of the trypan blue solution before being pipetted onto proliferating preadipocytes grown on 12-well plates in 1 ml medium per well. After incubation for 3 minutes, cells were counted under the microscope at 10x magnification, thereby comparing viable unstained cells to blue-stained necrotic cells.

2.5 Determination of differentiation

2.5.1 Morphological analysis

Morphological cell analyses to determine the extend of preadipocyte differentiation were performed using a phase-contrast microscope at a 10x magnification, counting cells in the field of vision, randomly chosen. Five fields were counted. Main morphological criteria for differentiation were an increasing number and size of visible lipid droplets as well as a change in morphology from elongated contours to a round shape.

2.5.2 Determination of differentiation by glycerophosphate dehydrogenase (GPDH) assay

To evaluate preadipocyte differentiation on a molecular level, changes in GPDH activity were measured according to a method by Wise and Green [172]. GPDH is a molecular key marker of adipogenic conversion which catalyzes the reversible conversion between dihydroxyacetone phosphate and glycerol 3-phosphate using NAD as a coenzyme. Glycerol 3-phosphate is one of the basic componends in triacylglycerol-formation when estered with long-chain fatty acids. GPDH activity is known to rapidly increase in the differentiation process from preadipocytes to adipocytes [173]. Changes in the absorption of the coenzyme NAD after enzymatic reaction can be measured by ELISA-reading at 340 nm which gives rise to GPDH activity. The used reagents derived from a kit system from Takara. Cells were washed twice with PBS and cell lysis was induced by adding "Enzyme Extraction Buffer". Afterwards, the lysate was diluted to two-fold by adding "Dilution Buffer". Enzyme activity was calculated with the following formula supplied by Takara: GPDH activity (units/ml) = OD_{340} x 2.06 x dilution ratio of the sample. Cellular protein content was measured according to a method by Bradford [174] to adapt GPDH levels to cell number.

2.5.3 Oil Red O staining

Oil Red O staining was performed according to a method by Zacarias et al. [175]. Monolayer cultures were washed with PBS and fixed with cold 10% paraformaldehyde and incubated for five hours at 4° C. Oil Red O working solution (0.5 g in 100 ml isopropanol) was added to culture flasks for two hours at room temperature. After washing, stained cells were kept in 10% paraformaldehyde and examined by light microscopy.

2.6 RNA isolation

Cells were cultured and differentiated in 6-well plates as described above. At day 10-14 of differentiation, RNA isolation was performed according to the "RNeasy Mini Handbook" from Qiagen: cells were washed twice with PBS, lysated with "RLT" buffer (after addition of 10 μl β-mercaptoethanol per 1 ml buffer), homogenized with a "QUIAshredder" spin column, and centrifuged at 10.000 G in a collection tube for 2 minutes. 600 μl of 70% ethanol were added before applying 700 μl of the sample to an "RNeasy" mini column placed in a collection tube and centrifuged for 15 seconds at 8000 G. The flow-through was discarded. 350 μl "RW1" buffer was pipetted into the mini column, and centrifuged for 15 seconds at 8000 G. To digest DNase, 10 μl "DNase I stock solution" was added to 70 μl "RDD" buffer. This incubation mix was pipetted directly onto the "RNeasy" silica-gel membrane, and placed on the benchtop for 15 minutes. Afterwards, 350 μl "RW1" buffer was pipetted into the mini column, centrifuged for 15 seconds at 8000 G; the flow-through was discarded. After transferring the RNeasy column into a new collection tube, 500 μl "RPE" buffer was added to the column. After centrifugation for 15 seconds at 8000 G, the flow-through was discarded. Another 500 μl "RPE" buffer were added to the tube which was centrifuged for 2 minutes at 8000 G. To elute the RNA, the column was placed into a new collection tube and 30-50 μl RNase-free water was given directly onto the column which was then centrifuged for 1 minute at 8000 G. All steps were performed using RNase-free pipette tips and RNase-free water according to the "RNeasy Mini Handbook". The RNeasy Kit and RNase-free DNase Set contained all of the listed buffers, reagents, and tubes.

2.7 Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

RT was carried out at 37° C for 60 minutes with oligo dT (15mer) as primer using the Omniscript-Kit. The cDNA (500 ng each) was used as template for PCR, primed by using oligonucleotides and conditions as shown in table 2.2. Extensive testing for each gene product was performed, to ascertain that amplification was always in the linear range. After amplification, PCR-products were subjected to electrophoresis on 1.8% agarose gels. Bands were visualized by ethidium bromide staining. Densitometric analysis of the visualized amplification products was performed using the GeneGenius documentation and analysis software (Syngene Cambridge, UK).

Table 2.2: Primer used for PCR.

Gene	primer sequence	ann. temperature	cycles
GAPDH	f: 5‘-CAACTACATGGTTTACATGTTCC-3‘ r: 5‘-GGACTGTGGTCATGAGTCCT-3‘	60°C	24
Mn-SOD	f: 5‘-AACGCGCAGATCATGCAGCTGC-3‘ r: 5‘-CCCAGTTGATTACATTCCAA-3‘	55°C	40
Cu,Zn-SOD	f: 5‘-ACGAAGGCCGTGCGTGCTGAA-3‘ r: 5‘-ACCACAAGCCAAACGACTTCCAGC-3‘	64°C	40

2.8 Statistical analysis

Data are given as arithmetical means ± SD. Statistical analyses were performed using GraphPad 5.0 statistical software (GraphPad Inc., USA). GraphPad InStat was used to calculate one-way ANOVAs to compare treatment variation, GraphPad Prism was used to calculate two-way ANOVAs to determine differences between treatments at certain time points. Given p-values are compared to standard proliferation or standard differentiation conditions, respectively, using Dunett's post-hoc test. Standard deviations (±) are given throughout.

3 Results

3.1 Toxicity of DETA/NO and DETA

To monitore the toxicity of DETA and DETA/NO on preadipoytes, cells were cultured as described above for 6 days. DETA/NO or DETA were added every 24 hours at 50-150 μM. The number of necrotic cells was analyzed every day by the trypan blue exclusion assay. As can be seen from Fig. 3.1, the mean number of dead cells in the untreated control is 9.9% $\pm$ 1.5%. Highest toxicity levels were found with DETA/NO at 150 μM (15.1% $\pm$ 2.6%), however, these values are not significantly different from control values. Toxicity of DETA/NO at a concentration of 100 μM and 50 μM was lower than found with 150 μM (10.2% $\pm$ 2.8 and 9.2 $\pm$ 1.7, respectively). DETA applied at 150 μM showed no toxicity (10.5 $\pm$ 2.1 necrotic cells) (Fig. 3.1).

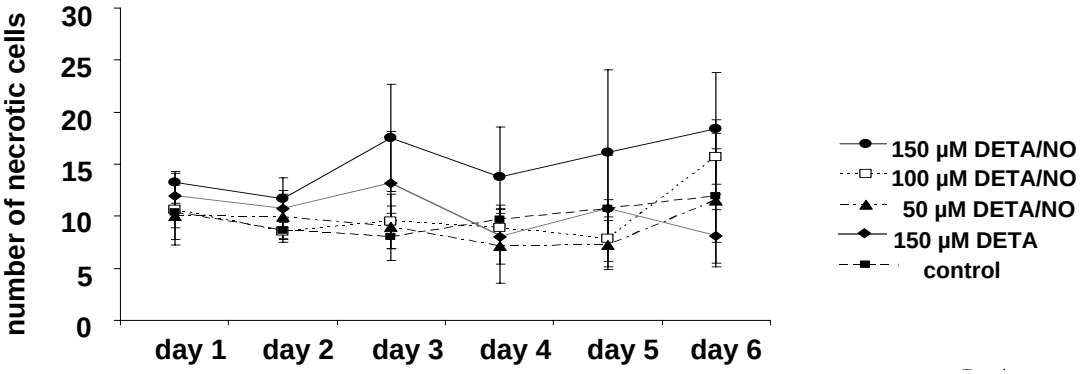

Figure 3.1: Toxicity of DETA/NO and DETA on preadipocytes. Preadipocytes were cultured as described. DETA/NO and DETA were added every day at concentrations indicated. The number of necrotic cells was analyzed every day by the trypan blue exclusion assay.

3.2 Effect of NO on preadipocyte proliferation and differentiation

In order to determine the effects of NO on proliferating preadipocytes, DETA/NO or DETA were added at 50 μM, 100 μM, or 150 μM. Proliferation was evaluated every day by cell counting. As shown in Fig. 3.2 A., there is an exponential growth in the control group. In all DETA/NO-treated cells proliferation was strongly inhibited with only small increases in cell number. This NO-mediated inhibition was concentration dependent with lowest cell numbers at 150 μM DETA/NO and highest cell numbers at 50 μM DETA/NO. Compared to the control, growth inhibition is significant at day 5 ($p \leq 0.05$) and at day 6 ($p \leq 0.001$) (Fig. 3.2 A.). In order to analyze whether the growth inhibitory effect was caused by NO or the donor molecule DETA, preadipocytes were cultured as described before for 7 days with daily addition of DETA (Fig. 3.2 B.). The XTT-assay confirmed the exponential growth of the control group. Addition of DETA does not inhibit the growth of preadipocytes but causes a slightly quicker proliferation than found in untreated cells. However, this difference to control treatment did not reach significance.
To analyze the effect of NO on preadipoyte conversion during differentiation, DETA/NO or DETA were added at concentrations indicated on confluent preadipocytes grown in differentiation medium. The extend of differentiation in the control group varies between 17.5% and 27.3% differentiated cells (compare Fig. 3.3 A.). With DETA, no change in the extend of differentiation was observed (Fig. 3.3 B.). In contrast, DETA/NO at 50 μM and 100 μM increased preadipocyte maturation significantly (46.7% $\pm$ 4.2% differentiated cells at 50 μM ($p \leq 0.01$) and 45.9 % $\pm$ 7.2 % differentiated cells at 100 μM ($p \leq 0.01$), compare Fig. 3.3 C.,D.). Concentrations higher than 100 μM revealed toxic effects on the cells after a few days. Analysis of GPDH was found to be in complete accordance with microscopical evaluations (Fig. 3.4). DETA/NO added at concentrations from 30 μM to 100 μM increased preadipocyte maturation. This increase was significant for DETA/NO from 50 μM to 100 μM ($p \leq 0.05$) with a peak at 70 μM (Fig. 3.4).

3.3 Molecular signaling pathway of NO in preadipocyte differentiation

To further determine the role of NO in preadipocyte differentiation and detect the underlying signaling pathway 70 μM DETA/NO was applied in combination with the guanylyl cyclase inhibitor ODQ, cGMP-analogues (8-Br-cGMP and 8-pCPT-cGMP (PDE-stable)),

the adenlyl cyclase inhibitor DDA, the adenylyl cyclase activator forskolin, and the cAMP-analogue 8-Br-cAMP (Fig. 3.5). Even though this concentration of NO had caused the strongest increase in adipogenic conversion (172.6% ± 35.2%), the presence of ODQ decreased the NO-mediated effect (33.5% ± 4.8% differentiated cells) below control levels (standard differentiation), and the combination of ODQ and DDA had an even stronger effect (22.1% ± 15.1%, $p \leq 0.05$). With DDA alone, differentiation is reduced to 47.97% ± 37.3%. NO addition antagonizes this effect in part (75.66% ± 15,95%). Applying the activators of guanylyl and adenylyl cyclase pathways together with the standard differentiation mixture, relative preadipocyte differentiation was increased to 187.8% ± 85.6% (8-Br-cGMP), 166.5% ± 24.8% (8-pCPT-cGMP), 188.63% ± 65.84% (forskolin), 204.79% ± 81.05% (8-Br-cAMP), and 182.51% ± 45.97% (8-Br-cAMP+8-Br-cGMP) compared to controls (set to 100%) (Fig. 3.5). With most activators of preadipocyte differentiation (8-pCPT-cGMP, forskolin, and 8-Br-cAMP), NO-addition caused an even higher extend of differentiation ($p \leq 0.05$) (see Fig. 3.5). The most effective combination to maximize preadipocyte differentiation was 8-Br-cAMP + NO (256.0% ± 38.5% differentiated cells). Interestingly, the combination of 8-Br-cGMP and NO did not promote differentiation (73.8% ± 26.0 differentiated cells) which might be due to degradation of 8-Br-cGMP by phosphodiesterases. When the phosphodiesterase-stable cGMP-analogue 8-pCPT-cGMP was applied in combination with NO, differentiation increased to to 191.9% ± 16.0% compared to standard differentiation ($p=0.02$). The combination of 8-Br-cGMP and 8-Br-cAMP also promoted differentiation and NO addition further increased the adipogenic conversion ($p \leq 0.01$).

3.4 Optimization of differentiation media

To facilitate culture conditions, the standard differentiation medium was modified with the aim to optimize culture conditions with the least amount of differentiation inducing factors. The standard differentiation medium consists of DMEM/Ham's F12 plus the 6 reagents IBMX, pioglitazone, dexamethasone, transferrin, insulin, and triiodo-L-thyronine (first 5 days, from then on without IBMX and pioglitazone). The number of factors was reduced in different set-ups and replaced by other differentiation-promoting reagents (8-pCPT-cGMP and 8-Br-cAMP). As expected, omitting of reagents led to decreased differentiation in test conditions without addition of cGMP/cAMP-analogues (see Fig. 3.6). As can be seen from Fig. 3.6, none of the tested conditions reached the same amount of differentiation compared to standard differentiation, despite addition of promoters of

preadipocyte differentiation. The combination that caused best results for differentiation on an average was IBMX and dexamethasone + 8-pCPT-cGMP but this result did not reach significance (see Fig. 3.6). Significant variation from controls ($p \leq 0.05$) was reached in IBMX + dexamethasone, IBMX + dexamethasone + insulin with and without addition of 8-Br-cAMP. Weakest differentiation results were reached with the combination of dexamethasone and insulin + 8-pCPT-cGMP (3,3% differentiated cells). Here, cytotoxic effects dominated in 2 of 3 patients although the concentration was the same as in standard differentiation medium (1 mM).

3.5 Effect of clozapine on preadipocyte proliferation

Preadipocytes were cultured in DMEM/F12 with FCS and bFGF supplementation for 6 days. Clozapine, LiCl, and EGCG were added at concentrations indicated. The XTT assay was applied after 2 and 4 days of proliferation. As can be seen from Fig. 3.7, growth rates did not vary significantly from controls in any of the test conditions. The XTT assay underlined the non-toxicity of the used reagents since each condition had about the same amount of viable cells at each measurement (day 0, day 2, day 4).

3.6 Effect of clozapine and EGCG on preadipocyte differentiation

To analyze the effect of clozapine and EGCG on preadipocyte conversion, differentiation was induced at cell confluence. During the first 5 days of differentiation, clozapine, propofol, LiCl, and EGCG were added at concentrations indicated. The extend of differentiation was determined morphologically (Fig. 3.8) and on a molecular level (Fig. 3.9). As shown in Fig. 3.8, controls (standard differentiation (A.), propofol (D.), and LiCl (E.)) showed an almost equal amount of differentiated cells as indicated by intracellular fat vacuoles which were seen in light microscopy (left column) and particularly marked after Oil Red O staining (right column). Conditions treated with clozapine (B., C.) showed a higher percentage of differentiated cells compared to controls, as confirmed by a larger number of intracellular fat vacuoles in both light microscopy and Oil Red O staining. Also, a slightly enhanced number of differentiated cells was found at 20 μM clozapine compared to 5 μM clozapine, indicating a concentration-dependent enhancement of differentiation. Fig. 3.9 reveals the activity of GPDH in % compared to standard differentiation. Standard differentiation was set 100% in order to standardize varying GPDH-activities from different

patients. Both clozapine concentrations (5 μM and 20 μM) demonstrate a significant and dose-dependent increase in GPDH-activity up to 30% ($p \leq 0.05$). Propofol and LiCl did not cause an increased GPDH-activity, they rather seemed to inhibit the differentiation process. However, this observation was not confirmed by morphological findings (compare Fig. 3.8). EGCG shows the most effective inhibition of GPDH-activity (approximately 85%, $p \leq 0.01$). Cells treated with EGCG in combination with clozapine still demonstrate a significant decrease in GPDH-activity compared to standard differentiation and clozapine only treatment ($p \leq 0.05$). This is supported by morphological findings, i.e. less vacuoles and elongated cell shape (Fig. 3.9).

3.7 Intracellular effects of clozapine

Analyzing the molecular impact of clozapine on the redox status in preadipocytes, we found no upregulation of Cu,Zn-SOD in the presence of clozapine but downregulation of Mn-SOD at 5 μM clozapine (Fig. 3.10). In the presence of EGCG and clozapine at the same time, however, expression of Mn-SOD was strongly upregulated whereas Cu,Zn-SOD expression was not increased (Fig. 3.10. LiCl induced both Mn-SOD and Cu,Zn-SOD which is consistent with the findings of Rodriguez de la Concepcion [176] in brown adipocytes.

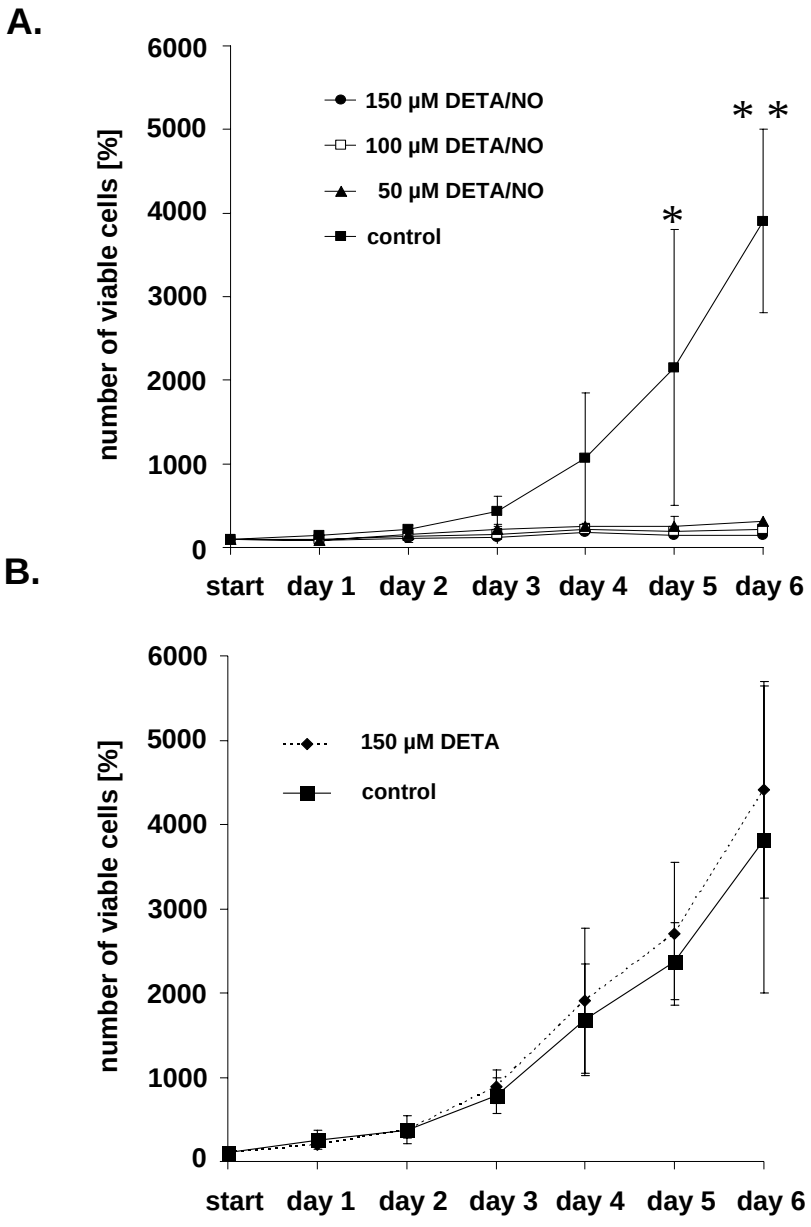

Figure 3.2: Preadipocyte proliferation under NO-influence. Preadipocytes were treated as described in Fig. 3.1. Instead of determining the amount of necrotic cells, proliferation was evaluated every day by trypsinising cells and counting them under the microscope in a Neubauer chamber (A.) or by performing XTT-assays (B.).

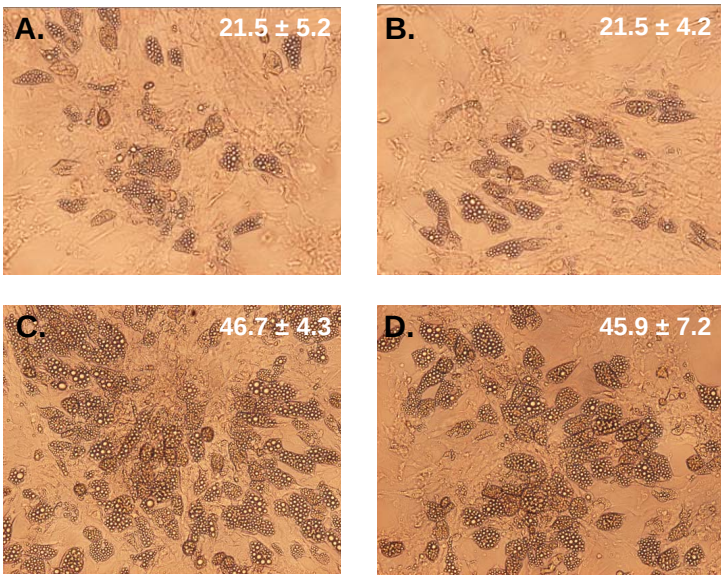

Figure 3.3: Morphological analysis of NO effects on preadipocyte differentiation. During the first 5 days of differentiation, DETA/NO or DETA were added at concentrations indicated to confluent preadipocytes grown in standard differentiation medium. Shown here is the morphological analysis of the influence of NO on preadipocyte differentiation after 14 days without the addition of DETA/NO (A.), in the presence of DETA (B.), after treatment with 50 μM (C.) and 100 μM (D.) DETA/NO. Given in the right top corner of every picture is the counted number of differentiated cells ($\pm$SD) as assayed after 15 days.

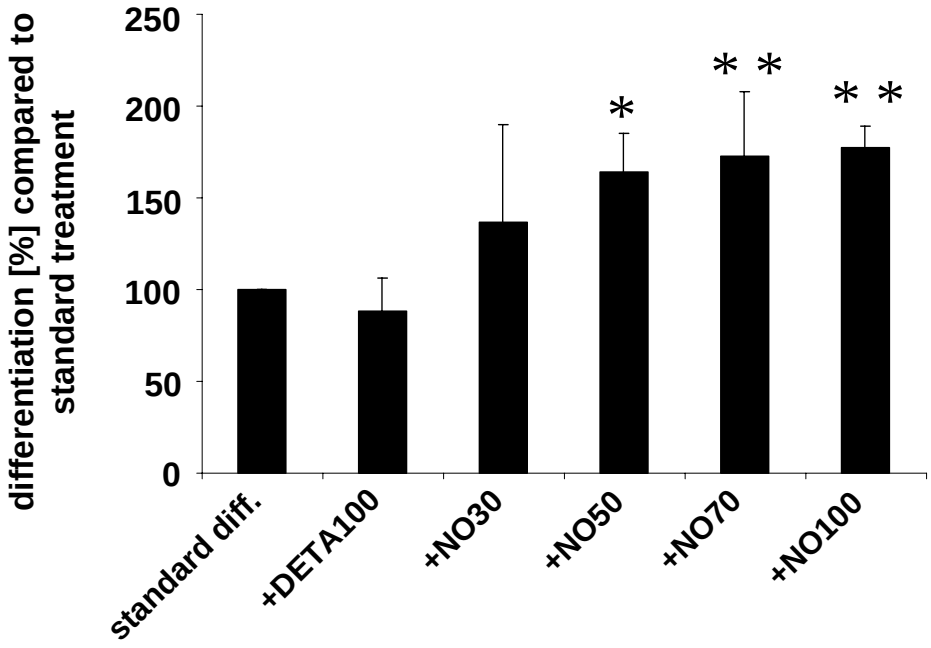

Figure 3.4: GPDH-activity under the influence of DETA/NO and DETA. To determine the effect of NO on preadipoyte differentiation on an enzymatical level, cells were cultured until confluence and differentiated as described above. During the first 5 days of differentiation, DETA/NO was added at 30 μM (+NO30), 50 μM (+NO50), 70 μM (+NO70), or 100 μM (+NO100) to the standard differentiation medium (standard diff.). DETA as control was applied at 100 μM (Fig. 3.4). Given percental values are relative to standard differentiation treatment which is set 100%. *, $p \leq 0.05$, **, $p \leq 0.01$

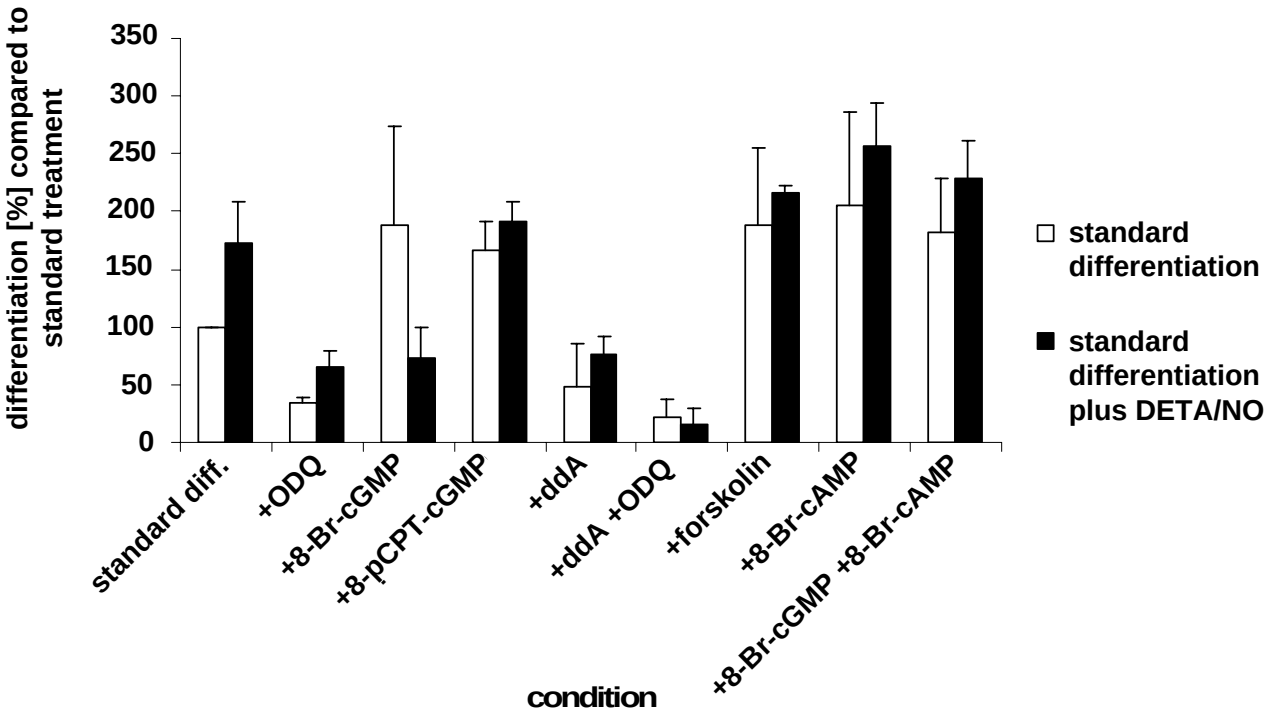

Figure 3.5: GPDH-activity under the influence of activators/inhibitors of guanylyl and adenylyl cyclase with and without DETA/NO-addition. To determine the ability of each reagent to increase or inhibit preadipocyte differentiation, cells were cultured until confluence and differentiated as described above. During the first 5 days of differentiation, all reagents except DETA/NO at 70 μM (+NO70) - which was added on a daily base - were added every second day to the standard differentiation medium (standard diff.). Given percental values are relative to standard differentiation treatment which is set 100%. All values varied significantly from the standard differentiation ($p \leq 0.05$) except for ODQ, ODQ + DETA/NO, 8-Br-cGMP + DETA/NO, 8-Br-pCPT-cGMP, DDA, DDA+ DETA/NO, and 8-Br-cGMP + 8-Br-cAMP.

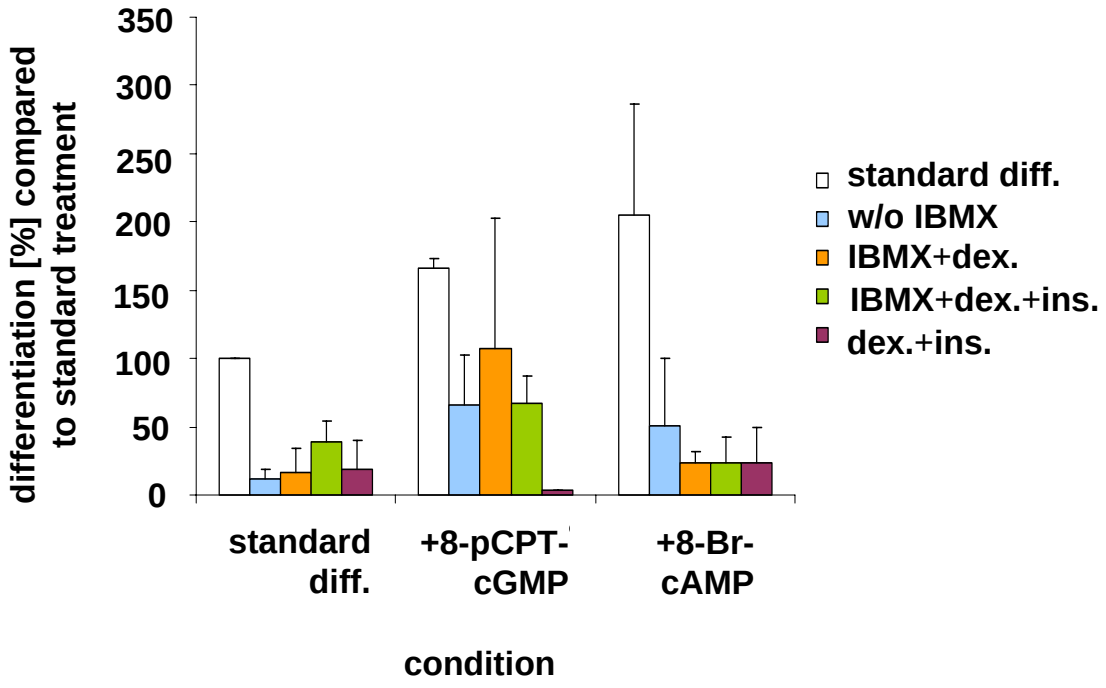

Figure 3.6: GPDH-activity of different culture media with or without addition of cGMP/cAMP-analogues. Preadipocytes were differentiated under standard conditions without addition of reagents (control) in standard differentiation medium or varied media as indicated in the legend. cGMP and cAMP-analogues (8-pCPT-cGMP and 8-Br-cAMP) were added at 1 mM every second day for the first 5 days of differentiation. Given percental values are relative to treatment in standard differentiation which is set to 100%.

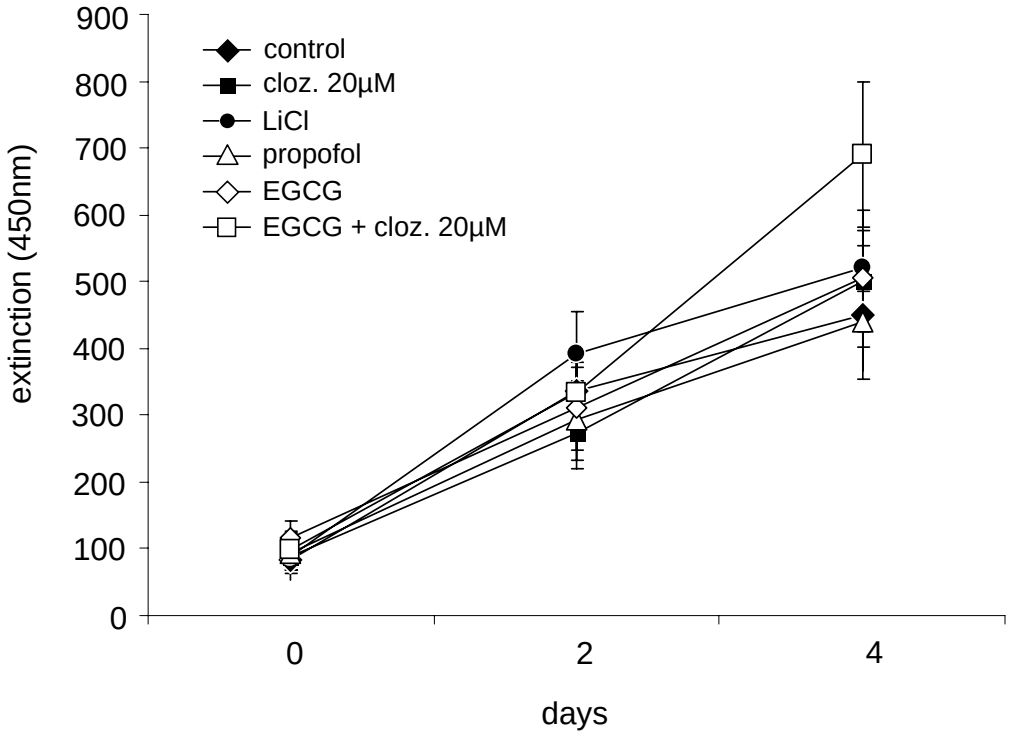

Figure 3.7: Preadipocyte proliferation under the treatment of clozapine, propofol, LiCl, and EGCG. Preadipocytes were grown in DMEM/Ham's F12 medium with 10% FCS, and bFGF, and clozapine, LiCl, and EGCG were added as concentrations indicated. The XTT assay was carried out on days 0, 2 and 4. Shown are results from 3 individual experiments.

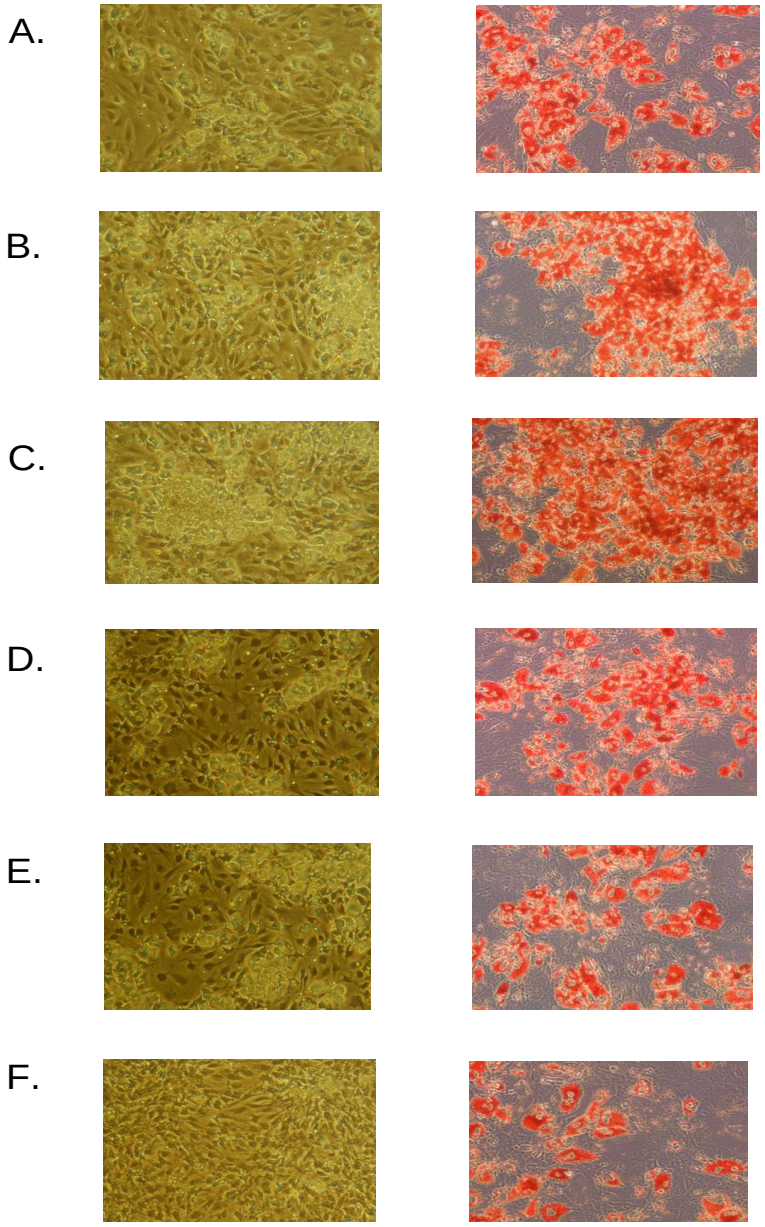

Figure 3.8: Morphological analysis of preadipocyte differentiation under clozapine treatment. To analyze the effect of clozapine on preadipocyte differentiation, cells were isolated, cultured until confluence and differentiated. Clozapine, propofol, LiCl, and EGCG were added as indicated. After 8-12 days, the extend of differentiation was determined by light microscopy in unstained (left column) or Oil Red O-stained (right column) cells. Shown are representative light microscopy images from three individual experiments. **A.** control, **B.** 5 μM clozapine, **C.** 20 μM clozapine, **D.** 10 μM propofol, **E.** 2 mM LiCl, **F.** 10 μM EGCG.

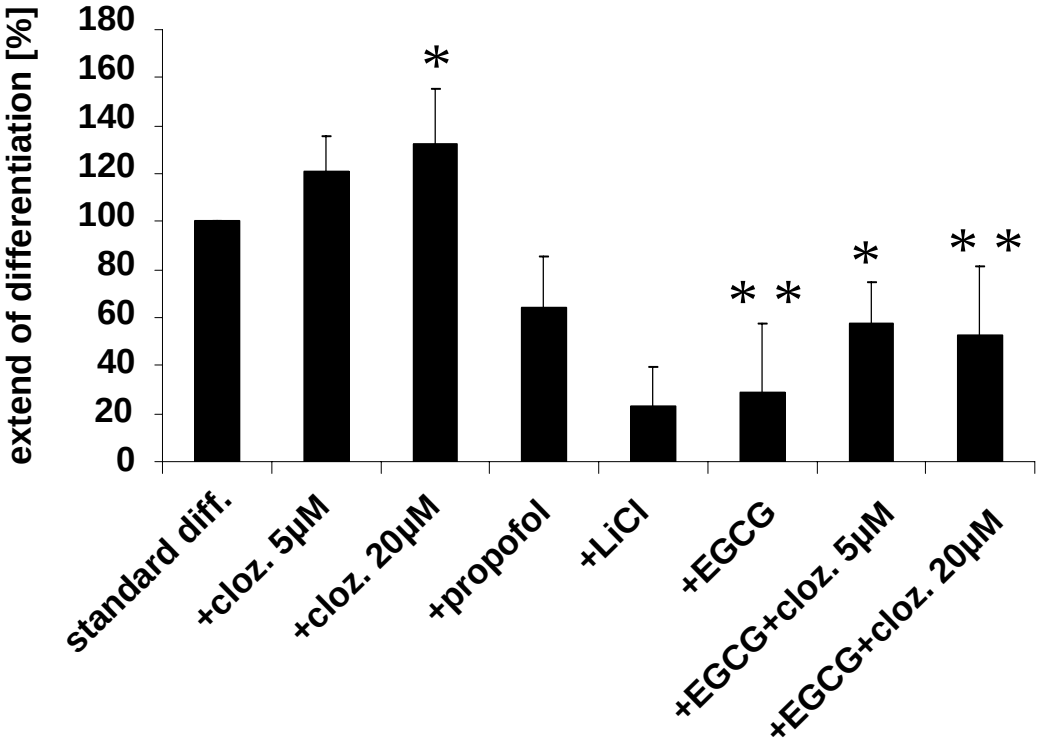

Figure 3.9: Molecular analysis of preadipocyte differentiation under clozapine addition. Cells were treated as described in Fig. 3.8. After 14 days of differentiation, the extend of preadipocyte conversion was determined by measuring GPDH activity relative to standard differentiation which was set 100%. Results are from 7 individual experiments. cloz. = clozapine, 5 μM and 20 μM; propofol = 10 μM; LiCl = 2 mM; EGCG = 10 μM *, $p \leq 0.01$ **, $p \leq 0.001$ compared to standard differentiation

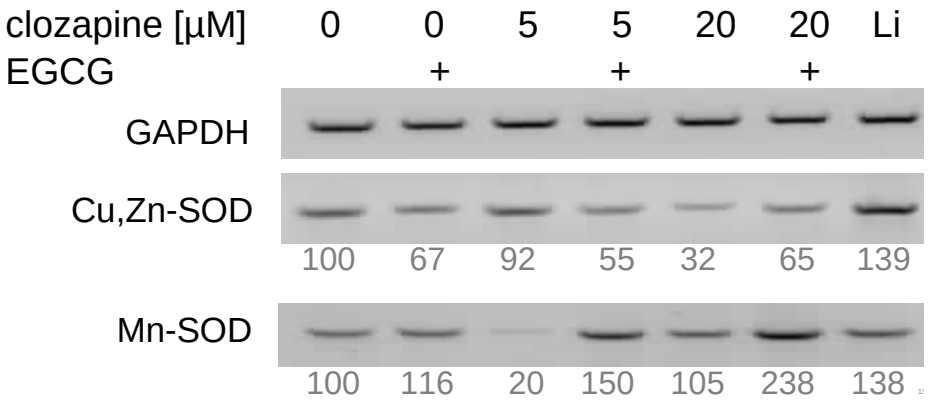

Figure 3.10: Molecular analysis of preadipocyte proliferation under clozapine and EGCG treatment. Preadipocytes were cultured as described before. The expression of Cu,Zn-SOD and Mn-dependent superoxide dismutase (Mn-SOD) was determined by RT-PCR under treatment with clozapine, EGCG, and LiCl. Shown is a representative agarose gel out of 3 individual experiments.

4 Discussion

4.1 Nitric oxide and adipogenesis

The role of nitric oxide in regulating biological functions including neurotransmission, blood vessel tone, host defense, and immunity has become the focus of intensive research throughout the last two decades [177, 178]. Its role in adipogenesis has been studied by several groups focussing mainly on lipolysis and obesity mechanisms [113, 179–181]. Looking at the key features of adipogenesis - differentiation and proliferation - only a few publications studied the effects of nitric oxide on differentiation and proliferation of human preadipocytes *in vitro*: Yan et al. observed a promoting effect of nitric oxide on differentiation in rat preadipocytes [107] which is consistent with the results in this thesis for human preadipocytes. However, an inhibition of preadipocyte proliferation was not found by Yan et al. in contrast to the findings in brown fat cells [182] and the results presented here. Looking at eNOS and iNOS expression in differentiating cells, Yan et al. confirmed the findings by Elizalde et al. [183], Ryden et al. [184], and Kikuchi-Utsumi et al. [185]: both isoforms were present in differentiating preadipocytes. A more recent study by Engeli et al. [115], however, found increased iNOS expression whereas eNOS expression was not influenced.

Another aspect of this thesis was the detection of the intracellular cascade of nitric oxide in preadipocytes. The main mechanism of intracellular NO-effects in most cells involves the cGMP-cascade (see 1.4.1) which has been presented for NO in brown adipocytes by Uchida et al. [165]. In brown adipocytes, NO is also known to up-regulate the expression of PPARγ [182]. Indeed, inhibition of guanylyl cyclase by ODQ caused a decreased differentiation of preadipocytes in this study (Fig. 3.5). Nevertheless, the extend of this inhibition is not able to explain the entire NO-effect whereupon we assumed other pathways might be involved, too. Apart from cGMP-independent pathways such as protein modification by S-nitrosation of thiol groups (see 1.4.1), pathways involving adenylyl cyclase could also be a target of pro-adipogenic factors: pharmacological studies in cultured cells show that, depending on the cell type examined, increased cAMP can exert opposite

effects in gene expression and regulation of transcription factors including CREB and C/EBP, being either stimulatory or inhibitory in adipocytes and other cells [186]. In this context, the primary mechanism of cAMP-mediated action involves modulating effects on iNOS-expression [186]. Since the main emphasis of this thesis was put on the effects of added NO-donating agents rather than intracellular NO-production, I focussed primarily on the potency of adenylyl-cyclase inhibitors such as 2'-5'-dideoxyadenosine (DDA)and guanylyl cyclase inhibitors such as ODQ to reduce the effects of extracellularily added NO. The inhibitory effects of DDA were not as distinct as with ODQ indicating that the main mechanism involves the guanylyl cyclase rather than adenylyl cyclase (Fig. 3.5). Addition of both inhibitors decreased differentiation to such a high extent that one has to consider a strong involvement of both pathways in adipogenesis in general (Fig. 3.5). However, the inhibitory effect of DDA could also be explained by the interruption of the cAMP-induced modelling effects on intracellular iNOS expression thus decreasing NO-release which could again promote differentiation via guanylyl cyclase. The exact role of intracellularily released NO in adipogenesis was not part of this study but is worth being studied further.

4.2 Inflammatory response

The above mentioned interrelations of nitric oxide and adipogenesis are also worth being discussed when considering inflammatory processes at the site of future implantation of preadipocyte-loaded scaffolds. Obesity is associated with inflamed adipose tissue, which can be seen on a molecular level by decreased expression of the anti-inflammatory adipokine adiponectin and increased secretion of a variety of proinflammatory cytokines, e.g. tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and prothrombotic factors such as plasminogen activator inhibitor-1 (PAI-1) [117,119,187]. Also, the increased mass of dysfunctional adipose tissue in obesity is known to be a source of several inflammatory factors, including TNF-α, IL-6, and PAI-1 [188–191]. These proinflammatory molecules have direct effects on adipose cell metabolism: TNF-α decreases insulin sensitivity and promotes lipolysis [192, 193]. Adiponectin, in contrast, improves insulin sensitivity and inhibits vascular inflammation [194–196]. TNF-α, IL-6, transforming growth factor beta (TGF-β), and interferon gamma (INF-γ) have been described to be strongly mitogenic for preadipocytes [197–200]. A more recent explanation of adipocyte-related inflammatory processes was given by Weisberg et. al in 2003: they first described the role of adipose tissue macrophages which strongly participate in inflammatory pathways [201]. The adipose

tissue of obese mice had an increased number of macrophages which were later demonstrated to impair adipogenesis via lipolytic activities and moreover seemed to produce the main fraction of inflammatory molecules in the adipose tissue [202, 203]. *In vitro*, inflammation induced by zymosan (a polysaccharide of the cell wall of saccaromyces cerevisiae bacteria [204]) does not only activate macrophages and neutrophils [205, 206], it has also been shown to interact with preadipocytes [207]. In macrophages, contact with zymosan particles activates NF-κB which leads to the production of cytokines. Hemmrich and Thomas recently showed that zymosan treatment of preadipocytes also led to increased TNF-α levels and promoted adipogenesis at an early stage [116]. The mechanism behind is believed to be linked to increased iNOS-expression. iNOS is one of the key effector enzymes in inflammation which is inducible by various inflammatory cytokines and is a downstream target of NF-κB [208–212] (see 1.4.2). Furthermore, Chung at al. assumed that preadipocytes are in truth responsible for the recruitment of monocytes/macrophages via MCP-1 and are moreover able to generate inflammatory signals thereby inhibiting PPARγ expression and insulin sensitivity in adipocytes [213]. Thus, there is a strong cross-link between preadipocyte and macrophage activation. Preadipocytes also express receptors for inflammatory chemokines [214] and migrate in response to them [215]. The above mentioned MCP-1 is one of these chemokines to which preadipocytes express receptors [214]. MCP-1 is secreted by mature adipocytes in obesity and the metabolic syndrome [58, 216, 217]. Hemmrich and Thomas just confirmed its adipogenesis-inducing abilities [116]: preadipocytes migrated in response to MCP-1 and other macrophage directing chemokines therefore allowing adipogenesis even in the absence of pre-existing fat. Although the maintenance of a chronic low-grade inflammation state and higher expression of iNOS, TNF-α, Il-6, and CRP in the adipose tissue are generally linked to the progression of obesity [117, 218–220], one has to be cautious to assume a general positive correlation between inflammation at the site of the transplant and adipogenesis: TNF-α, IL-6, transforming growth factor β (TGF-β), and interferon γ (INF-γ) have been shown to inhibit preadipocyte differentiation and lipid accumulation [197, 198, 200, 221, 222]. Another hypothesis regarding the cross-link between inflammation and obesity is the notion that inflammation can be seen as an adaptive response to hypoxia in the expanding fat tissue [223]: hypoxia causes HIF-1-mediated PPARγ repression (see 1.3.2) which limits further adipogenesis. Hemmrich and Thomas also observed inhibitory processes due to inflammation in the final phase of adipogenesis [116]. In this study, however, the proinflammatory molecule NO strongly promoted adipogenesis. Since NO shows a high diversity in influencing intracellular pathways, one cannot exclude that it plays an exceptional role in preadipocyte differentiation. These results have been confirmed by the

findings of Hemmrich and Thomas [116]: they observed reduction of adipogenic response of over 50% when NO-production was blocked. Inflammation at an early stage of adipogenesis even induced *de novo* endogenous adipogenesis and preadipocyte migration.
These observations have to be brought to mind when future transplantations of adipose tissues are planned. Inflammation at the site of the transplant might be detrimental or beneficial, depending on the amount and composition of inflammatory molecules. Further *in vivo* research is therefore necessary. A possible application of NO-donors can however stimulate preadipocyte differentiation. Nevertheless, the inhibitory effect of NO on preadipocyte proliferation should be considered when applied at an early state of cell proliferation: the NO-effect might be detrimental for a transplant without complete cell congruency. If preadipocytes ought to be seeded onto three-dimensional carriers, they should therefore undergo a long proliferating phase until being attached to other cells before NO addition. Imaginable would be NO-addition at a state of cell congruency *in vitro* or after implantation *in vivo* therefore allowing a more effective differentiation process especially in larger transplants. However, the positive effect of NO on vessel formation [224–226] (see section 4.3) would have to be taken into consideration when outweighing the advantages and disadvantages of late NO application.

4.3 "Side-effects" of NO: promotion of angiogenesis and cell survival

Another limitation of current adipose tissue engineering applications is the insufficient vascularization and hypoxic conditions of the three-dimensional constructs, especially in the center. Although NO-effects on endothelial cells were not part of this study, this topic has to be considered in the context of improving and understanding angiogenesis. The key molecule in angiogenesis, as well as in vasculogenesis during embryonic development, is VEGF which can be induced by a number of angiogenic stimuli such as cytokines, hormones, and hypoxia [227]. Most embryonic vessels and endothelial cells express receptors for VEGF. In the last 10 years, more and more evidence emerged that VEGF and VEGF-related pathways are also inducible by NO-donors [102, 228]. Yet, only a certain amount of NO will upregulate VEGF gene expression while an excessive dose of NO inhibits VEGF expression via unidentified pathways [229]. Furthermore, VEGF may activate eNOS expression in endothelial cells [230]. The resulting NO will then in turn mediate the angiogenic effects of VEGF [224–226]. This VEGF-mediated NO synthesis is due to an immediate pathway via phosphorylation of eNOS and a delayed effect

causing both phosphorylation and upregulation of eNOS [231–234]. Apart from VEGF, expressional increases by NO have also been shown for bcl-2 expression at the mRNA and the protein level [235–239]. Bcl-2, a stress response gene, is a potent apoptosis onset inhibitor [238]. The balance between the anti- and pro-apoptotic proteins of the bcl-2 family of proteins regulate cell death and cell survival. The exact mechanisms are still unclear but some groups relate them to the ability of the bcl-2 family proteins to form ion channels and complexes with other proteins [240–244]. The most common hypothesis regarding the cell stabilizing effect of bcl-2 involves prevention of mitochondrial cytochrome c release [245–248]. This model was also confirmed by Hemmrich et al. in 2003 [249] in antisense-mediated iNOS-knockdown in endothelial cells: these cells were more susceptible to reactive-oxygen species-mediated cell death which elucidates the pivotal role of NO in bcl-2 expression. A more recent study by Hida et al. in 2004 [250] combines the role of NO in the expression of VEGF and bcl-2: their in vitro results confirm the promoting effect of NO on endothelial cell proliferation and angiogenesis as well as its protective ability against apoptogenic agents. Summarizing these observations, NO-donating agents - when used with care - may not only enhance the differentiation of adipogenesis in the implant but also improve vascularization and resistance against hypoxic conditions by upregulating VEGF and bcl-2 expression.

4.4 Optimization of preadipocyte culture conditions

A thorough understanding of the differentiation process of preadipocytes is necessary to improve and facilitate culture conditions. Fig. 1.8 summarizes effectors and possible mechanisms of adenylyl and guanylyl cyclase-dependent cascades. Although Ibrahimi et al. [251] postulate a decreased differentiation process under elevated intracellular cAMP-levels, most studies agree to the pro-adipogenic potential of cAMP [163, 164]. Its potency to induce CREB has already been described by Roesler et al. [252] about 20 years ago. A more recent study underlines the pivotal role of CREB in initiating preadipocyte differentiation [164]. Moreover, cAMP itself seems to activate the expression of stearoyl-CoA desaturase gene 1 thereby stimulating this fatty acid-modifying enzyme which is involved in differentation by producing monosaturated fatty acids from saturated precursors [163]. Yet, the role of cAMP in adipogenesis and lipolysis seems to be paradoxical: some laboratories found increased lipolysis after stimulation of cAMP-PKA [253–255] and even decreased obesity in PKA knockout-mice [256]. These controversial results may be explained by the complex mechanisms of cAMP on the one hand and by different study designs on

the other hand: cAMP and PKA regulate various intracellular systems (not just CREB) and, vice versa, CREB may not alone be influenced by cAMP but by numerous growth factors [257, 258]. cAMP-PKA signaling may also depend on the stage of adipocyte differentiation: other PPARγ agonists such as FK614 (3-(2,4-dichlorobenzyl)-2-methyl-*N*-(pentylsulfonyl)-3-*H*benzimidazole-5-carboxamide) have differentiation stage-dependent effects which contribute to an enhanced insulin sensitization in differentiating adipocytes and to reduced insulin resistance at the stage of adipocyte hypertrophy [259]. In conclusion, although cAMP induces differentiation from preadipocytes to adipocytes, its effect on lipid metabolism in mature adipocytes may be totally different. The results of this study support the pro-adipogenic model of cAMP in early differentiation: the adenylyl cyclase activator forskolin as well as cAMP-analogues (8-Br-cAMP) and inhibitors of PDEs (IBMX) enhanced differentiation to a high extent (Fig. 3.5). All reagents were added for the first 5 days of differentiation where they seemed to have the best differentiation-stimulating abilities, which is consistent with the above mentioned interrelations.
Besides cAMP, cGMP and NO also present potent stimulators of differentiation. The intracellular actions of NO are described more thoroughly in the section 4.1. In this context, the guanylyl-cyclase dependent pathway is of special interest. cGMP and its potency to enhance preadipocyte differentiation has rarely been studied. Phosphorylation of protein kinases, however, plays a major role in regulating transcription factors in other cell types [260–262], which also includes activation of CREB [263]. Thus, both adenylyl and guanylyl-dependent pathways may end in similar phosphorylation activities, at least to some extent (see also Fig. 1.8) which explains their synergistic effects shown in this study (see Fig. 3.5). A combination of NO with activators of adenylyl cyclase and analogues of cGMP and cAMP resulted in an even faster differentiation process. Nevertheless, one should be cautious with combinations of all reagents since overstimulation often caused poorer differentiation especially in more susceptible cells (as could be observed in some samples that derived from liposuction which were not included in this study). My observations can be helpful in tissue engineering in terms of *in vitro* enhancement of differentiation before transplantation of adipose tissue grafts or even *in vitro* using cGMP- or cAMP-donating agents. By omitting certain reagents in the culture medium and replacing them with more potent reagents (NO, forskolin, and analogues of cAMP or cGMP), the whole culturing process can be facilitated in general which might also be helpful for further research on preadipocytes in other contexts. Unfortunately, most combinations resulted in poorer differentiation-processes compared to the standard differentiation medium (Fig. 3.6). Thus, it remains questionable if this type of medium variation will prevail in preadipocyte culturing since it led to rather cytotoxic effects in some patients

which might explain the high standard deviation and low significance. IBMX seemed to have the strongest differentiation-promoting effect since ommitting of this reagent led to poor results for differentiation (see Fig. 3.6) whereas addition often caused overstimulation and cytotoxy in combination with cGMP/cAMP-analogues. These results are controversial at times, since a combination of IBMX, insulin, and cGMP/cAMP-analogues always led to cytotoxic effects even though the concentration of all reagents was not changed. Also, the combination of IBMX, dexamethasone, and insulin together with these activators showed weaker GPDH-activities than the combination of IBMX and dexamethasone alone. Thus, the particular balance of activators of differentiation might be an underestimated factor. Maybe a reduction of IBMX-concentration in the media could present a basis for further research since the cells seemed to be stressed (seen by changed cell shape) in test conditions with IBMX without "outbalancing" factors as can be found in the standard differentiation medium. I therefore conclude that the most effective differentiation was still observed in standard differentiation medium with added promoters of differentiation such as cGMP/cAMP-analogues, forskolin, and NO. If time is more important, I would recommend continuing preadipocyte differentiation under these circumstances. If incomplex experiment set-ups are needed - as in adipose tissue engineering - a modified medium containing DMEM/Ham's F12, IBMX and dexamethasone plus cAMP or cGMP analogues could represent an adequate alternative to the standard if IBMX-concentration is reduced (follow-up experiments needed).

4.5 Effects of clozapine and green tea on adipogenesis

Weight gain is a common complication of antipsychotic treatment starting a few months after initiation of therapy [127]. Common hypotheses for the pro-adipogenic effect of clozapine involve CNS-based neuroendocrine circuits regulating the interaction of leptin, ghrelin, and adiponectin. All three hormones play a major role in the regulation of energy homeostasis and food intake [133, 264]. However, so far, results concerning clozapine-related weight gain remain controversial: Atmaca et al. observed significant increases of plasma triglyceride and leptin levels in patients treated with clozapine suggesting a positive correlation between leptin and weight gain [132]. In contrast, Albaugh et al. found lowered plasma levels of glucose and leptin in rats under treatment with olanzapine whereas clozapine did not show this effect [265]. Also, some studies aimed at explaining the clozapine-induced weight gain by increased plasma levels of insulin, ghrelin, and adiponectin: in their cross-sectional study, Togo at al. found no significant increase

in serum ghrelin levels in patients treated with clozapine compared to healthy individuals [134]. The same observation was made by Theisen et al. [266] in a prospective study whereas Hosojima et al. [135] report a decreased serum ghrelin level in patients treated with olanzapine and clozapine as well as unchanged adiponectin and insulin plasma levels. These discrepancies might be explained by different study designs, application of different neuroleptics, different lengths of observation, and numbers of patients. Still, on the basis of all literature reports, there is no definite correlation between weight gain and plasma levels of hormones regulating energy homeostasis. Another focus of attention in the context of neuroleptics-induced weight gain are genetic polymorphisms as found for various ligands, such as second messengers coupled to the serotonin (5HT) receptor [267] or direct binding of atypical neuroleptics to receptors that regulate weight [265]. Again, these studies have not yet found a plausible explanation for weight gain in patients. The fact, that even strict diets are unable to stabilize body weight points towards other mechanisms to be the cause of the observed weight gain. This study suggests fat neo-formation as the key mechanism for weight gain. I found a significant increase of 30% in preadipocyte differentiation under clozapine treatment compared to controls. I propose that the effect of clozapine on preadipocyte differentiation relies on the intracellular localization of the drug. Clozapine preferentially accumulates in lipid membranes where it acts as an excellent radical scavenger [268].

The redox metabolism in cells corresponds to a complex interacting network involving the generation of reactive oxygen species and cellular antioxidant defenses (see also chapter 1.3.3) [84]. Any disturbance of this balance can induce several transduction pathways and transcription factor activities, e.g. erythropoietin and vascular endothelium growth factor gene transcription [74, 84].

Carriere et al. demonstrated that propofol induces preadipocyte differentiation by radical scavenging [74, 83]. I propose the same effect for clozapine. Fig. 4.1 summarizes the proposed interrelations between ROS production, clozapine, and EGCG. Increased mitochondrial ROS production can be eliminated by upregulation of Mn-SOD. This seems to be exactly the pathway, EGCG uses to counteract clozapine-induced differentiation. In clozapine-treated preadipocytes, Mn-SOD expression is significantly induced in the presence of EGCG (Fig. 3.10) and the resulting hydrogen peroxide inhibits further differentiation (Fig. 4.1). EGCG-mediated upregulation of Mn-SOD and subsequent hydrogen peroxide production has previously been observed in cancer cells which contain higher levels of superoxide [162, 269]. Thus, EGCG has pleiotropic effects with mechanisms of action that include working as an antioxidant or a pro-oxidant [269]. In the presence of clozapine, EGCG appears to function as a pro-oxidant that induces NF kappa

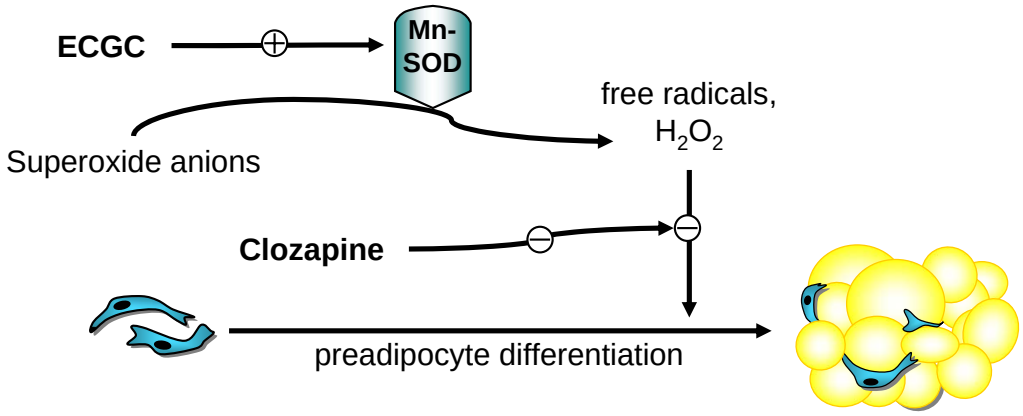

Figure 4.1: Proposed scheme of the influence of clozapine and EGCG on adipogenesis. Clozapine promotes radical scavenging leading to enhanced preadipocyte maturation. EGCG inhibits clozapine-enhanced differentiation by induction of Mn-SOD. The resulting product H_2O_2 inhibits preadipocyte differentiation.

B activation [270] and subsequent Mn-SOD expression. In line with my results that Mn-SOD activity prevents preadipocyte differentiation, Lechpammer et al. [271] reported that Mn-SOD-/- bone marrow stromal cells demonstrate constitutive adipocytogenesis and generate 34% more adipocytes in differentiation medium than Mn-SOD+/+ cells. Moreover, Mn-SOD polymorphisms have been described, that might contribute not only to clozapine-involved weight gain. Recently, Galecki et al. reported a Val-9Val genotype of the Mn-SOD gene that is associated with a three times higher risk for schizophrenia [272]. This mutation influences the mitochondrial targeting of the enzyme and leads to enhanced oxidative stress associated with schizophrenia [273]. I speculate that the beneficial effects of clozapine treatment are at least partially based upon its radical scavenging capacity. Nevertheless, the intracellular mechanisms of oxidative stress are complex processes involving cell-specific coping with superoxide anions and other free radicals. In contrast to Carriere et al. and contrary to my assumption, propofol did not increase differentiation of preadipocytes by decreased hydrogen peroxide levels and free radical scavenging. This observation might be due to different susceptibility of differentiating human preadipocytes to propofol (in contrast to preadipocyte cell-lines as being used by Carriere et al.) and different experimental set-ups (8-12 days of differentiation prior to GPDH-assay compared to 96 hours in the set-up of Carriere et al.). My results might be more significant since the differentiation process was assessed for a longer time-interval

and was documented morphologically, too. Nevertheless, this fact does not explain the controversial issue around oxidative stress and propofol, the most supposable explanation could be the neglectable propofol-stimulus in the complex mechanism of preadipocyte differentiation in my experiments whereas clozapine presented a stronger stimulus. The influence of oxidative stress on fat cell formation is seen controversially in the literature, too: clinical studies postulate a correlation of decreased plasma antioxidants with obesity prevalence [274] suggesting that oxidative stress promotes adipogenesis. Moreover, Fujita et al. associated increased systematic oxidative stress with visceral fat accumulation and metabolic syndrome [275]. Nevertheless, these studies regarded systemic markers rather than intracellular redox changes and are therefore less concerned with processes directly linkable to the adipose tissue. Also, this study is not the first to describe clozapine-treated preadipocytes: in 2003, Hauner et al. investigated the effects of clozapine on mammary adipose tissue and found no increased fat cell formation [276]. Neither proliferation nor differentiation of preadipocytes was enhanced under clozapine-treatment. The fact that these findings are contradictory to mine might be explained by the attributes of the mammary tissue which was the only tissue source used by Hauner et al. and the low number of tested tissue specimens. My adipose tissue samples were derived from abdominoplasty operations since abdominal fat cells have a higher susceptibility to hormonal stimulation and are therefore more suitable for research on preadipocyte differentiation [277, 278]. However, the *in vitro* enhancement of preadipocyte differentiation of approximately 30% does not completely explain the immense weight gain in patients. Considering intraindividual differences in long-term studies [131], multifactorial influences such as genetic predisposition and comorbidity might also contribute to the extent of weight gain. Still, my results present an easy and effective way to prevent the clozapine-induced weight gain to some extent. EGCG in green tea or in form of capsules could counteract the enhancement of differentiation since my *in vitro* results suggest that it effectively prevents preadipocyte differentiation by upregulating Mn-SOD(Fig. 3.10), causing higher concentrations of hydrogen peroxide. EGCG is also known to reduce levels of adipogenesis-related transcriptional factors, such as C/EBPβ and PPARγ [158], and to suppress the activation of enzymes responsible for lipid metabolism, e.g. sterol regulatory element binding protein-1 (SREB-1), in adipose tissue [159]. Various studies show that EGCG reduces size and number of lipid droplets and GPDH-activity in differentiating preadipocytes [160]. Although clinical data have so far shown little potency of EGCG on weight reduction in obese people, in vitro studies and animal trials have repeatedly demonstrated its anti-adipogenic effect [158–160]. Recent studies on olanzapine-induced weight gain undertaken in the USA [126] and China [279] reveal a medium weight gain

of 15.4 ± 10 kg in American people versus 8.34 ± 5.97 kg in Chinese patients after 2 years of treatment. This striking discrepancy may indicate that the high consumption of green tea in China is involved in the decreased weight gain under therapy with atypical neuroleptics. Thus, EGCG presents an easily applicable and promising tool in treating clozapine-induced weight gain by counteracting clozapine-mediated preadipocyte differentiation and by general inhibition of lipid metabolism. Moreover, due to the general beneficial effect of green tea on health, EGCG treatment may be useful to prevent the metabolic disturbances often leading to the metabolic syndrome under clozapine treatment [160].

5 Conclusion

The findings of this study extend the understanding of how NO plays a significant role in regulating proliferation and differentiation of human preadipocytes. This is helpful to modify present tissue engineering applications in order to improve cell maturation and organization in three-dimensional biohybrids by applying nitric-oxide donating agents *in vitro* and *in vivo*. Furthermore, the findings on optimizing and facilitating preadipocyte culture may be useful in further research on adipogenic precursors, in tissue engineering as well as in reasearch on obesity or adipose-tissue related diseases: the culture medium was modified to such an extent that the differentiation process from preadipocytes to mature fat cells was shortened from 14 days to 8-10 days on an average. An alternative culture medium was also presented in using the formerly applied reagents of the standard medium substituted with potent activators of adenylyl- or guanylyl-cyclase-dependend pathways thereby facilitating experimental set-ups. In a second study, the intracellular mechanisms of clozapine and EGCG on preadipocyte proliferation and differentiation were evaluated. The findings support the hypothesis of a proadipogenic clozapine effect which can be counteracted by EGCG. This is most likely due to the radical scavenging abilities of clozapine which favour preadipocyte differentiation probably independent from receptor-activated pathways. The proadipogenic effects of clozapine lead to an enlarged fat mass which is detrimental in clozapine-therapy since it impairs health status and compliance of treated patients. EGCG could be a potential remedy by up-regulating Mn-SOD and inhibiting preadipocyte differentiation in general. Drinking green tea might therefore be an easy but effective way to reduce the clozapine-induced weight gain. This study gives an example of possible mechanisms but might not completely explain the entire extent of clozapine-induced enhancement of preadipocyte differentiation. That is why further studies are needed to outline alternative intracellular mechanisms elucidating the complex mechanism of intracellular clozapine-effects more thoroughly.

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List of publications

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K. Hemmrich, C. Gummersbach, N. Pallua, C. Luckhaus, K. Fehsel. Clozapine enhances differentiaation of adipocyte progenitor cells, *Mol Psychiatry 2006 Nov;11(11):980-981.*

Book article

K. Hemmrich, G. P. L. Thomas, C. Gummersbach, K. M. Abberton, A. J. Penington, W. A. Morrison. Inflammation causes neo-adipogenesis - what adipose tissue engineering can learn from the pathogenesis of obesity. *Chirurgisches Forum 2007, 36, pp. 303-304, ISBN 3-540-71122-8, Springer*

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K. Fehsel, C. Gummersbach, K. Hemmrich, N. Pallua, C. Luckhaus. Clozapine enhances differentiation of preadipocytes. *European Archives of Psychiatry and Clinical Neuroscience 2006; 256; supp. 2: II 37*

K. Hemmrich, C. Luckhaus, C. Gummersbach, N. Pallua, K. Fehsel. Clozapine enhanced differentiation of preadipocytes. *Int. J. Neuropsychopharmacology 09/2006 (Suppl. 1): S209*

Oral presentations

K. Hemmrich, G. P. L. Thomas, C. Gummersbach, K. M. Abberton, A. J. Penington, W. A. Morrison. 124. Congress of the German Society of Surgery, Munich, Germany 04/2007: Entzündung verursacht die Neubildung von Fett - was die Fettgewebszüchtung von der Pathogenese der Fettleibigkeit lernen kann

K. Hemmrich, M. Wosnitza, C. Gummersbach, D. von Heimburg, N. Pallua. Bernard O'Brian Institute of Microsurgery Alumni Meeting, Melbourne, Australia 03/2007: The use of preadipocytes for adipose tissue engineering

C. Gummersbach, K. Hemmrich, N. Pallua, K. Fehsel. 7th Leiden International Medical Students Conference, Leiden, Netherlands 03/2007: Green tea extract presents new options to manage weight gain under psychopharmacotherapy.

K. Fehsel, C. Gummersbach, K. Hemmrich, N. Pallua, C. Luckhaus (2006). Congress of the German Society of Biological Psychiatry, Munich, Germany, 10/2006: Clozapine enhances differentiation of preadipocytes.

K. Hemmrich, G. P. L. Thomas, C. Gummersbach, K. Abberton, E. W. Thompson, N. Pallua, W. A. Morrison. European Conference of Scientists and Plasti Surgeons (ECSAPS) London, England 09/2006: Role of iNOS and NO in adipose tissue engineering

K. Hemmrich, C. Luckhaus, C. Gummersbach, N. Pallua, K. Fehsel. Collegium internationale neuro-psychopharmacologicum (CINP) congress Chicago, USA 07/2006: Clozapine enhanced differentiation of preadipocytes

C. Gummersbach, K. Hemmrich, N. Pallua, K. Fehsel. X. Maastricht Medical School Research Conference, Maastricht, Netherlands 03/2006: Enhanced differentiation of progenitor fat cells - a possible mechanism of clozapine-induced weight gain

C. Gummersbach, K. Hemmrich, M. Meersch, N. Pallua. IX. Maastricht Medical Students Research Conference, Maastrich, Netherlands 03/2005: Optimized adipose tissue engineering with the use of nitric oxide-donating agents

Poster presentation

C. Gummersbach, K. Hemmrich, M. Meersch, N. Pallua, Chirurgische Forschungstage Munster, Germany 09/2006: Preadipocyte differentiation is enhanced by the signalling molecules nitric oxide , cGMP and cAMP - new options for old hurdles in adipose tissue engineering

Abstracts

Optimized adipose tissue engineering with the use of nitric oxide-donating agents

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Introduction: Nitric oxide (NO) has multiple effects on cellular pathways of human cells. This study focusses on the impact of NO in adipose tissue engineering and inflammatory processes after transplantation of soft tissue grafts. These grafts represent an adequate option to correct soft tissue defects resulting from deep burns, tumor resections or congenital defects. Analyzed in this study are the effects of NO on the proliferation and differentiation of preadipocytes, progenitor cells with stem-cell characteristics, within the adipose tissue. The aim is to optimize adipose tissue engineering and to anticipate possible complications due to inflammatory reactions in wound situations after transplantation.
Materials & Methods: Preadipocytes were isolated from human subcutaneous adipose tissue samples and cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) with 10% fetal calf serum (FCS). Cells were grown in bFGF (basal fibroblast growth factor)-supplemented medium with the NO donor molecule DETA/NO added at various concentrations for proliferation and differentiation. Differentiation was induced by insuline, isobuthylmethylxanthine, pioglitazone, dexamethasone and transferrin after 7-14 days of culture expansion and in absence of FCS. Proliferation was analyzed by microscopy and by XTT-tests which determine the absorbance of a formazan dye which can only be produced by viable cells. Differentiation of preadipocytes was assayed after 15 days by cell counting and analysis of the enzyme glycerophosphate dehydrogenase which is highly expressed in mature adipocytes.
Results: Our results show a significant inhibition of preadipocyte proliferation in the presence of NO. Further, we observe an enhanced maturation of precursor cells to mature fat cells in the presence of DETA/NO. NO also increases the number of differentiated cells if adipogenic conversion is only promoted by dexamethasone, insuline and transferrin.
Discussion & Conclusion: Our results emphasize the important role of NO in tissue engineering and are encouraging for applying NO-donors during transplantation of preadipocytes in a three-dimensional setting since it helps optimizing differentiation of adipogenic precursor cells. Thus, NO-supplemented implants open new perspectives for adipose tissue engineering to treat extended soft tissue defects.

Enhancement of differentiation of progenitor fat cells: a possible mechanisms of clozapine-induced weight gain

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Introduction: Psychoactive drugs and especially clozapine cause massive weight gain in patients. Recent studies focussing on the activation of neuropeptides which are involved in the appetite regulating network presented controversal results. We postulated a direct effect of clozapine on fat cells. Therefore we studied the influence of clozapine on progenitor fat cell differentiation to mature adipocytes leading to increased fat mass. Moreover, we tried to find out if extract from green tea (EGCG) which is known to inhibit the formation of triglyceride vesicle formation could prevent the clozapine-induced differentiation process.
Materials & Methods: Preadipocytes were isolated from human subcutaneous adipose tissue samples and cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) with 10% fetal calf serum (FCS). Differentiation was induced by insulin, isobuthylmethylxanthine, pioglitazone, dexamethasone and transferrin after 7-14 days of culture expansion and in absence of FCS. Clozapine, EGCG and LiCl were added every second day for the first 5 days of differentiation. LiCl, a mood stabilising drug, served as control since it induces weight gain as well but through different mechanisms such as glucocorticoid elevation. Differentiation was assayed after 8-15 days by cell counting and analysis of the enzyme glycerophosphate dehydrogenase highly expressed in mature adipocytes. We also examined the effects of oxidative stress, the rate of apoptosis and PCR analysis.
Results: Treatment of preadipocytes with clozapine induced a significant rise in GPDH activity and an elevated number of differentiated cells. Lithium chloride showed no difference compared to standard differentiation. Extract from green tea (EGCG) inhibited differentiation in general but also under clozapine treatment.
Discussion & Conclusion: Our results suggest a new explanation of weight gain in patients treated at least with clozapine: increased fat mass due to enhanced differentiation from progenitor fat cells to mature adipocytes. EGCG could help preventing this weight gain. We speculate that the enhanced differentiation might be caused by oxidative stress or the involvement of cGMP.

Green tea extract presents new options to manage weight gain under psychopharmacotherapy

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Introduction: Atypical neuroleptics such as clozapine and olanzapine have essentially improved the treatment of schizophrenia. Unfortunately, both drugs lead to an excessive weight gain in patients which reduces their compliance and also bears a risk for their physical health. Trying to find an explanation for the clozapine-induced weight gain, many studies presented controversial results for hormones involved in energy homeostasis, such as leptin or insulin. Focussing on a completely different aspect, we examined the effect of clozapine on the differentiation process from adipogenic precursor cells, called preadipocytes, to mature fat cells. We further studied possible intracellular mechanisms to find an effective remedy. Since cellular redox status and free radical formation are potent regulators of adipogenic conversion, we looked at the ability of extract from green tea (EGCG) to inhibit preadipocyte differentiation, thereby counteracting the pro-adipogenic effect of clozapine.
Methods: Preadipocytes were isolated from human subcutaneous adipose tissue samples and cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) with 10% fetal calf serum (FCS). Differentiation was induced by insulin, isobuthylmethylxanthine, pioglitazone, dexamethasone, and transferrin after 7-14 days of culture expansion and in absence of FCS. Clozapine, EGCG and LiCl were added every second day for the first 5 days of differentiation. LiCl, a mood stabilising drug, served as control since it induces weight gain as well but through different mechanisms such as glucocorticoid elevation. Adipogenic conversion was determined by measuring GPDH activity and Oil Red O staining. Cellular redox status was determined by superoxide radical formation and PCR-analysis of enzymes involved in reactive oxygen species formation (ROS).
Results: We found that clozapine-enhanced fat neo-formation is based on the ability of clozapine to scavenge free radicals, therefore reducing oxidative stress which favours preadipocyte differentiation. Green tea extract (EGCG) effectively inhibited clozapine-enhanced differentiation by strong induction of a key regulator in ROS-formation, Mn-superoxide dismutase (Mn-SOD). Mn-SOD converts superoxide anions to hydrogen peroxide which inhibits further differentiation from preadipocytes to mature fat cells.
Conclusions: Our findings indicate that the long-lasting, constant clozapine-induced weight gain is due to the powerful radical scavenging capacity of clozapine. This leads to an enhanced differentiation process from immature fat cells to adipocytes resulting in an enlarged fat mass. EGCG was able to efficiently prevent this detrimental process and could therefore present a promising tool to avoid weight gain under therapy with atypical neuroleptics.

Preadipocyte differentiation is enhanced by the signaling molecules nitric oxide, cGMP and cAMP - new options for old hurdles in adipose tissue engineering

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Background: This study aimed at analyzing the effects of nitric oxide (NO) on preadipocytes in a setting as found after transplantation of a biohybrid composed of viable adipose precursor cells and a three-dimensional matrix. Such an implant potentially represents an adequate solution for the correction of soft tissue defects, e. g. extensive deep burns or tumor resections. The function of NO in such a setting of adipose tissue generation has not been monitored yet. Besides its important role in regulating physiological and pathophysiological functions throughout the body, NO represents an early consequence and marker of proinflammatory reactions as found in wound situations after transplantation. A present focus of attention in tissue engineering is the insufficient maturation of preadipocytes which results in cell necrosis and fibrotic tissue formation, especially in the center of larger three-dimensional constructs. A transplanted preadipocyte-loaded scaffold with these attributes will not meet the requirements for modelling and refilling extensive soft tissue defects. Optimizing preadipocyte culture and differentiation is therefore crucial to overcome complications at the site of the transplantation. In this study, human preadipocytes were treated with the NO donor DETA/NO and its down-stream signalling molecules cGMP and cAMP to analyze the signalling pathway of NO in preadipocytes and to optimize adipogenic conversion to mature fat cells.
Methods: Preadipocytes were isolated from human subcutaneous adipose tissue samples and cultured in Dulbecco's modified Eagle medium (DMEM)/ Ham's F12 with 10% FCS. At confluence, differentiation was induced by insulin, pioglitazone, dexa-methasone, isobutylmethylxanthine, and transferrin in the absence of FCS. To further identify the molecular pathway, by which NO mediates its effect, the specific guanylyl cyclase inhibitor ODQ, the adenylyl cyclase inhibitor 2'-5'-dideoxy-adenosine, the cGMP and cAMP-analogues 8-Br-cAMP and 8-Br-cGMP, and the adenylyl cyclase activator forskolin were added. Proliferation was evaluated by microscopy and XTT-assays while differentiation was assessed by cell counting and analysis of the enzyme glycerophosphate dehydrogenase.
Results: Our results demonstrate that NO in combination with the conventionally used differentiation-inducing factors significantly (p¡0.01) enhances maturation of precursor cells to adipocytes via guanylyl cyclase dependent pathways but also - to a certain extend- via adenylyl cyclase dependent pathways. The combination of NO with activators of adenylat cyclase and cGMP-/cAMP-analogues results in a significantly accelerated and enhanced differentiation process (p¡0.05). Proliferation, in contrast, is inhibited in the presence of NO.
Conclusion: Our results help to understand the possible effects of inflammation in a

preadipocyte-seeded tissue construct. NO-release as a consequence of inflammatory processes will be beneficial at cellular confluence to substantially induce preadipocyte differentiation. Before confluence is reached, though, an inflammatory stimulus might be detrimental as it strongly inhibits preadipocyte proliferation. Our findings are encouraging for applying NO-donors and activators of cAMP/cGMP-dependent pathways during transplantation of preadipocytes in a three-dimensional setting to accelerate and optimize differentiation of preadipocytes. Furthermore, the results are helpful for modifying present tissue engineering applications in order to improve progenitor cell maturation and cell organization in three-dimensional biohybrids.

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Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten bei mir, ***Caroline Gummersbach, Roermonder Str. 139, 52072 Aachen,*** hinterlegt sind.

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