UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

O USO TERAPÊUTICO DE MEDIADORES ANTI-INFLAMATÓRIOS DA VIA DO ÁCIDO ARAQUIDÔNICO

Aluno: Carlos Antonio Trindade da Silva

Orientador: Prof. Dr. Carlos Ueira Vieira

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ALUNO: Carlos Antonio Trindade da Silva

COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Carlos Ueira Vieira

Examinadores: Dr. Carlo Jose Freire de Oliveira
Dr. Siomar de Castro Soares
Dra. Cássia Regina da Silva
Dra. Lara Vecchi

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Prof. Dr.Carlos Ueira Vieira

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15d-PGJ_2= 15-deoxi-$\Delta^{12,14}$-Prostaglandina J_2

AA = ácido araquidônico

COX = Ciclooxigenase

DHET= ácidos dihidroxieicosatrienoicos

EET= ácidos epoxieicosatrienoicos

IL-1β = Interleucina 1 β

IL-6= Interleucina 6

LOX = Lipoxigenase

LT= Leucotrieno

MCP-1= Proteína quimiotática de monócitos-1

NF-Ķb= Fator nuclear-κB

PLA_2= Fosfolipase A_2

PGE_2= Prostaglanina E_2

OPG= Osteoprotegerina

PERK= PKR- like endoplasmic reticulum kinase

PPAR = Receptor de Ativação de Proliferação de Peroxissomo

RANK/RANKL= Receptor ativador do ligante fator nuclear-kB

TNF-α= Fator de Necrose Tumoral α

sHE= Epoxido hidrolase solúvel

XBP-1 X-box binding protein 1

RESUMO

O ácido araquidônico (AA) é precursor na formação dos eicosanoides, que são mediadores lipídicos com uma série de funções na fisiologia e patologia humana. A maioria dos eicosanoides atuam como mediadores pró-inflamatórios e contribuem para o desenvolvimento e proliferação de tumores. Nesta tese foram avaliados dois mediadores: a 15-deoxi-$\Delta^{12,14}$-PGJ_2 (15d- PGJ_2) e os ácidos Epoxieicosatrienoicos (EETs), ambos apresentam uma atividade oposta a da maioria dos eicosanoides, ou seja, com uma ação anti-inflamatória e antitumoral. Esses dois mediadores distintos da via do AA foram utilizados nesta tese em dois projetos distintos. Primeiro: A 15d- PGJ_2 possui uma atividade antiproliferativa e induziu apoptose para diversos tipos de células tumorais, entretanto, o efeito da15d- PGJ_2 em células de cancer da tireoide ainda estava desconhecido. Neste sentido, foram cultivadas *in vitro* células tumorais da tireoide, da linhagem TPC1, e tratadas com diferentes concentrações de 15d- PGJ_2 (0 ate 20 μM), as células tratadas demonstraram uma diminuição na proliferação, e aumento na apoptose, e uma diminuição na liberação e expressão relativa de IL-6. Estes resultados em conjunto sugerem que a 15d- PGJ_2 pode ser utilizada como uma nova terapia para o cancer da tireoide. Segundo: Os EETs são metabolizados em seus diois pela epóxi hidrolase solúvel (sEH), para manter a estabilidade dos EETs e a sua atividade antiiflamatoria, foi utilizado um inibidor (TPPU) para sEH em um modelo de periodontite induzida por *Aggregatibacter actinomycetemcomitans*. O tratamento oral com TPPU, assim como o uso de animais sEH Knockout, levou a uma redução na perda óssea acompanhada da diminuição de moléculas osteoclastogenicas como RANK, RANKL e OPG, demonstrando que a inibição farmacológica da sEH pode ter um valor terapêutico na periodontite e doenças inflamatórias que envolvem a reabsorção óssea.

Palavras chave: Prostaglandinas, epóxi hidrolase solúvel, ácidos epoxieicosatrienoicos, doença periodontal, câncer de tireoide.

ABSTRACT

Arachidonic acid (AA) a precursor in the formation of eicosanoids which are lipid mediators with a number of functions in human physiology and pathology. The most of the eicosanoids act as proinflammatory mediators and contribute to the development and proliferation of tumors. In this thesis we evaluated two mediators: 15-deoxy-Δ12,14-PGJ2 (15d- PGJ2) and epoxieicosatrienoic acids (EETs) both act with an opposite activity of most eicosanoids, with an anti-inflammatory and and anti-tumoral action these two distinct mediators from AA pathway were used in this thesis in two different projects. First: 15d- PGJ2, was described that to have an antiproliferative activity and to induce apoptosis in several types of tumor cells however, the effect of 15d- PGJ2 in thyroid cancer cells was unknown in this sense, we tested *in vitro* cultured thyroid tumor cells, here in TPC1 cells, and treated with different concentrations of 15d- PGJ2 (0 to 20 uM) the treated cells showed a decrease in proliferation and an increase in apoptosis and a decrease in IL-6 release and relative expression. These key results together demonstrate that 15d- PGJ2 can be used as a new therapy for thyroid cancer. Second: The EETs are converted to their diols by soluble epoxy hydrolase (sEH) to maintain the stability of EETs and their anti-inflammatory activity, an inhibitor (TPPU) against was used to sEH in a periodontitis model induced with *Aggregatibacter actinomycetemcomitans*. The oral treatment in mice with TPPU and sEH Knockout animals showed bone loss reduction accompanied by a decrease in the osteoclastogenic molecules, like RANK, RANKL and OPG, demonstrating that pharmacological inhibition of sEH may have therapeutic value in periodontitis and inflammatory diseases that involve bone resorption.

Keys words: Prostaglandin, Epoxide Hydrolase soluble, Epoxieicostrienoic acid, Periodontal Disease, Thyroid Cancer.

APRESENTAÇÃO GERAL

A via do ácido araquidônico (AA) resulta na síntese de mediadores lipídicos indispensáveis na fisiologia e patologia humana. Dentre estes mediadores, chamados de eicosanoides, encontram-se as prostaglandinas, leucotrienos e os ácidos epoxieicosatrienoicos que desempenham diversas funções bioquímicas nas células, agindo como mensageiros de curta distância, com ação autócrina e parácrina.

Nas últimas décadas, diversos esforços têm sido feitos para o controle e modulação da via do ácido araquidônico, culminando no uso terapêutico das ações dos eicosanoides com o intuito de prevenir ou tratar uma serie de patologias, como a hipertensão arterial, artrite reumatoide, carcinomas e doença periodontal.

A 15-deoxi-$\Delta^{12,14}$-prostaglandina J_2 (15d-PGJ_2), é um ligante do Receptor Ativado por Proliferadores de Peroxissoma ɣ, esta prostaglandina possui diversas ações biológicas, como a capacidade anti-inflamatória e antitumoral. Neste sentido o seu uso exógeno tem sido utilizado para modular a resposta inflamatória, e também tem demonstrado um potencial terapêutico para inibir o crescimento de tumores como: mama, bexiga, pulmão e próstata, estes efeitos foram eficientes tanto em modelos experimentais *in vivo* quanto *in vitro*, no entanto o seu efeito sob o câncer de tireoide ainda está desconhecido, neste sentido foi avaliado o efeito *in vitro* da 15d-PGJ_2 em células da linhagem TPC1, no câncer da tireoide.

Os ácidos epoxieicosatrienoicos (EETs), são sintetizados a partir do AA por enzimas da via do citrocomo P450. Os EETs possuem diversas ações biológicas como vaso dilatadores, anti-inflamatórios e analgésicos. No entanto os EETs são rapidamente metabolizados pela enzima Epoxido Hidrolase solúvel (sEH), que converte os epóxidos dos EETs para os dióis, os ácidos dihidroepoxidoeicostriecos (DHET). Desta forma os EETs têm uma meia vida curta, dificultando o seu uso farmacológico, porém, neste sentido nos últimos anos foram desenvolvidos inibidores para a sEH a fim de aumentar a meia vida dos EETs, desta forma potencializando os seus efeitos biológicos.

A doença periodontal é uma doença inflamatória crônica que acomete cerca de 70% da população adulta. Mediadores inflamatórios gerados na doença periodontal ativam osteoclastos, que promovem a reabsorção óssea.

O periodonto é constituído por ligamentos, gengiva e osso alveolar. Estas estruturas promovem a fixação dos dentes na boca. A doença periodontal é caracterizada pela destruição destas estruturas. Sendo assim a modulação da resposta inflamatória na doença periodontal tem sido alvo, no intuito de reduzir a perda óssea, que se não for tratada pode levar a perda dos dentes.

Diante da potencial inibição da sEH, foi hipotetizado que o uso de inibidores da sEH para manutenção de elevados níveis de EETs, o que traria efeitos anti-inflamatórios, teria um efeito benéfico para evitar a perda óssea causada pela doença periodontal.

CAPÍTULO 1.
Fundamentação teórica

1. Ácido Araquidônico

O AA é oriundo do ácido linoleico, um ácido graxo essencial. A partir da formação do AA, esta molécula passa a constituir os fosfolipídios, que são componentes da estrutura da membrana das células. Cada fosfolipídio é constituído de uma molécula de glicerol que interliga a um grupo fosfato e dois ácidos graxo. Desta forma, o AA é um dos ácidos graxos que compõem a membrana celular (LEHNINGER & NELSON 2006).

Para iniciar a síntese da cascata do AA, é necessário que este ácido graxo esteja livre da membrana da célula. O processo de liberação do AA depende da ação de enzimas que são capazes de liberar o AA da membrana para o citoplasma. A fosfolipase A_2 (PLA_2), é a principal enzima responsável pela liberação do AA da membrana das células. No entanto, a clivagem do AA a partir do diacilglicerol pela diacilglicerol lipase, é uma via alternativa relacionada com a produção de endocanabinóides (LEHNINGER & NELSON 2006).

A PLA_2 é ativada pela fosforilação, que ocorre em resposta a eventos de transdução de sinal desencadeados por vários estímulos, como, por exemplo, a ação da trombina nas plaquetas, do C5a nos neutrófilos, da bradicinina nos fibrobrastos, reações antígeno anticorpo nos mastócitos e lesão celular em geral, leva a ativação da fosfolipase A_2, requer uma ação catalítica dependente de fosforilação, liberando o AA para o citoplasma da célula. O AA livre no citoplasma pode ser direcionado e metabolizado por três vias diferentes, a via da ciclooxigenase, lipoxigenase e do Citocromo P 450 (ONI-ORISAN, et al. 2016, MRUWAT, et al 2015).

1.2 Via das Ciclooxigenases

A COX tem duas isoformas, cada uma é originada de um gene diferente. O gene codificador da COX1 constitutiva, é localizado no cromossomo 9 e seu

produto (enzima COX 1) promove a regulação de diversas funções, como a proteção da mucosa gastrointestinal de ulceração. O gene codificador da enzima COX2 induzível, é localizado no cromossomo 1 e sua expressão é rapidamente aumentada em resposta a uma variedade de citocinas pró-inflamatórias e lesões teciduais (BACCHI, et at 2012). A enzima COX1 participa da síntese das prostaglandinas responsáveis pela citoproteção gastrointestinal e função plaquetária, enquanto a enzima COX2 induz a síntese de prostaglandinas, como por exemplo a prostraglandina E_2, que é responsável por induzir dor e inflamação (KRAEMER et al 1992; SIMMONS et al 2004). As duas isoformas, tanto a da COX-1 quanto a COX-2 ligam-se ao retículo endoplasmático promovendo uma ação de endoperóxido sintase, pela catalize da incorporação de duas moléculas de oxigênio ao araquidonato levando à uma ciclização para produzir o endoperóxido cíclico (PGG_2). Em seguida as COXs tem uma ação de peroxidase que converte a PGG_2 em outro endoperóxido cíclico, a PGH_2 (HANG et al. 2008). A PGH_2 é convertida através de várias sintases especificas de cada célula nas principais prostaglandinas como: prostaglandina sintetase converte para prostaglandina I_2, (PGI_2), a prostaglandina E_2 sintetase converte para prostaglandina E_2 (PGE_2), a tramboxano-A sintetase converte para Tramboxano A_2 (TXA_2) e a prostaglandina D_2 sintetase converte para prostaglandina D_2 (PGD_2); (figura 1) a PGD_2 sofre uma desidratação e espontaneamente é formada a prostaglandina J_2, Em seguida esta molécula sofre um rearranjo intramolecular formando a Δ^{12}-PGJ_2 que perde mais uma molécula de água formando a 15-deoxi-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (SCHER, PILLINGER, 2004; LAMANO-CARVALHO, 2007)

A 15d-PGJ_2 é produzida por uma variedade de células, incluindo macrófagos, mastócitos, células T e plaquetas (STRAUS *et al*, 2000). A maioria das prostaglandinas tem uma meia vida curta variando entre 30 segundos à 3 minutos (SWEETMAN, 2005). Embora pouco esteja estabelecido sobre a farmacocinética e estabilidade da 15d-PGJ_2, acredita-se que sua meia vida seja curta devido à conjugação de glutationa (GSH), o que é conhecida como uma importante via metabólica de prostaglandinas ciclopentônicas. (ATSMON et al., 1990; STRAUS et al., 2000) e que podem rapidamente ser eliminadas através do metabolismo do pulmão, fígado, plasma e excreção renal (HAGENS et al., 2007).

Durante o início do processo inflamatório, no qual encontramos um aumento do influxo de polimorfonucleares, há um aumento da atividade da COX2 e síntese de PGE_2, a qual possui um papel proinflamatório intenso. No entanto, aparentemente, a COX2 participa da resolução da fase aguda da inflamação alternando o padrão da síntese de prostaglandina para a família J. (SURH et at 2011). Além da fase aguda da inflamação, a PGE_2 está envolvida também na inflamação crônica e no desenvolvimento de tumores malignos (OBERMAJER et al 2012).

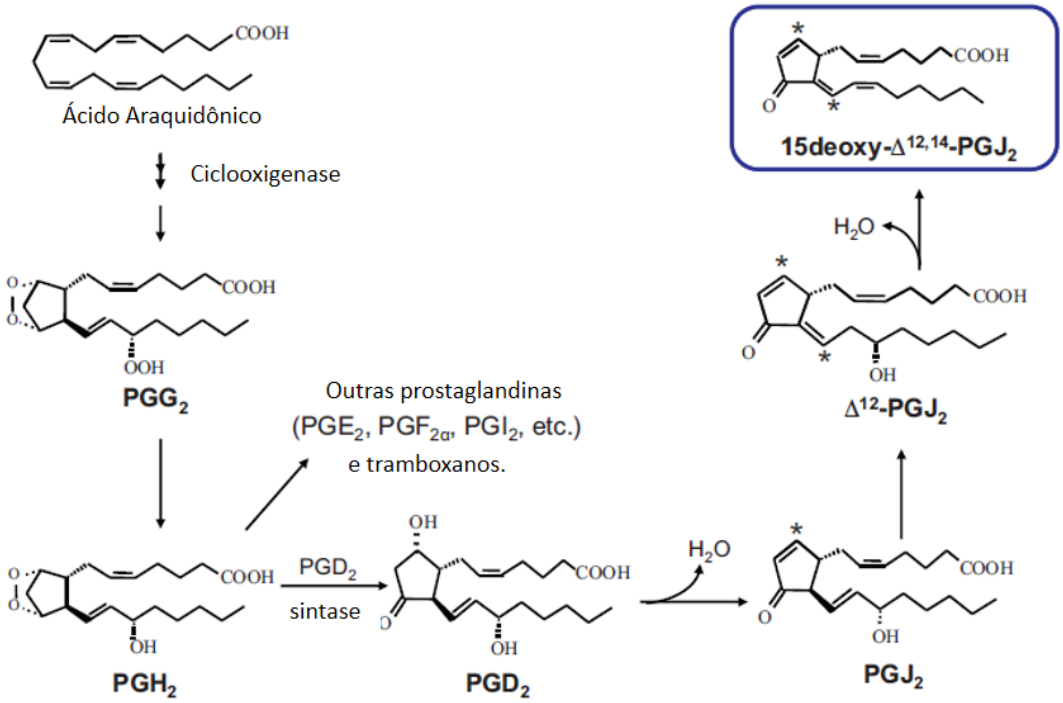

Figura 1. Síntese da 15d-PGJ_2, modificado e adaptado de Surh et al. 2011.

1.2.1 *15-deoxi-Δ^{12,14}-PGJ$_2$*

Recentemente, tem sido dada grande importância no papel da 15d-PGJ_2 na regulação do processo inflamatório e no desenvolvimento de tumores (JIANG, TING, SEED, 1998; RICOTE et al., 1998; CLARK et al., 2000, LIU et al 2014), devido à possibilidade de intervenção farmacológica ser aplicada na regulação da produção de citocinas e prostaglandinas. A 15d-PGJ_2, é um dos derivados do metabolismo da PGD_2, e um ligante natural (do inglês Peroxisome proliferator-

activated receptor gamma) PPAR-γ (FORMAN et al., 1995; RICOTE et al., 1998), o que sugere que esta molécula possui um importante papel na regulação da reposta inflamatória e no desenvolvimento de diversos carcinomas.

A subfamília de receptores nucleares PPAR consiste de três diferentes isoformas, PPAR-α, PPAR-β/δ e PPAR-γ, as quais são codificadas por diferentes genes além de possuírem diferentes promotores (ZHU et al., 1995). As três isoformas possuem padrões distintos em relação à distribuição celular e tecidual. O PPAR-γ foi descrito inicialmente expresso em adipócitos e hepatócitos, porém atualmente, sabe-se que o PPAR-γ é encontrado em macrófagos/monócitos, miócitos, fibroblastos e células precursoras de medula (BRAISSANT et al., 1996).

Acredita-se que o PPAR-γ em estado inativado esteja conjugado com proteínas co-repressoras, localizado no citoplasma ao invés do núcleo (BISHOP-BAILEY, HLA, 1999). A ligação do PPAR e a 15d-PGJ_2, induz a dissociação do PPAR de seus repressores, e permite a interação com co-ativadores (por exemplo; receptores esteróides), o que resulta na translocação deste do citoplasma para o núcleo (Zhu et al., 1997). O resultado desta translocação é a ativação da expressão ou repressão de uma variedade de genes que estão envolvidos no processo inflamatório e com o desenvolvimento e progressão de tumores (NEGISHI, KATOH, 2002, MORIAI et al 2009).

Napimoga et al. 2008 demonstrou que a 15d-PGJ_2 (ligante natural dos receptores PPAR-γ) inibe a migração de neutrófilos para o sítio inflamatório inibindo a expressão de ICAM-1 (do inglês Intercelular Adhesion Molecule 1) no endotélio sendo esta ação dependente da via do óxido nítrico. Por outro lado, a 15d-PGJ_2 também foi capaz de inibir a dor inflamatória devido à liberação de opióides locais.

Devido a sua capacidade eletrofílica a 15d-PGJ_2 pode se ligar a várias proteínas do meio extracelular como, por exemplo, na albumina que é a proteína encontrada em maior concentração no plasma. Experimentos *in vitro* demonstram que mais de 90% da 15d-PGJ_2 é inativada no meio extracelular, sendo assim, um pequeno percentual da 15d-PGJ_2 administrada tem uma atividade intracelular (OH et al. 2008).

A capacidade eletrofílica da 15d-PGJ_2 ocorre no anel ciclopentônico, através da reação de Michael, que consiste na adição nucleofílica de um carbânion ou outro nucleófilo a um composto carbonil α,β insaturado, que prontamente reage com substâncias que contenham grupos nucleofílicos, como cistenil tiol de proteínas. A reação seletiva da adição tiol ocorre na posição endocíclica, dentro do anel ciclopentônico (UCHIDA, CHIBATA, 2008).

Como citado anteriormente, devido a sua característica eletrofílica, quando administrada de forma exógena sua maior parte é perdida ao se ligar a proteínas do soro como a albumina, sendo rapidamente degradada. Alves et al 2011, na tentativa de modular esta característica eletrofílica da 15d-PGJ_2 desenvolveu um novo sistema de liberação sustentada em nanopartículas poliméricas biodegradáveis. Neste trabalho, a 15d-PGJ_2 apresentou um efeito 33 vezes mais potente, quando comparada a molécula livre, na diminuição da migração de neutrófilos e produção de citocinas próinflamatórias em um modelo de peritonite induzida. Ainda utilizando este modelo de liberação sustentada, Napimoga et al 2012, demonstrou que usando baixas doses de 15d-PGJ_2 hove uma inibição da reabsorção óssea em um modelo de periodontite induzida. Ainda envolvendo o metabolismo ósseo em processos patológicos, foi investigado a atividade da inibição da perda óssea associada com metástase de câncer de mama e a deficiência de estrógeno causada pelo tratamento de câncer. A 15d-PGJ_2 preveniu a destruição trabecular do osso femoral, atuando de forma benéfica para o tratamento de metástases ósseas associadas com o câncer de mama (DIERS et al 2010)

1.2.2 *15-deoxi-$\Delta^{12,14}$-PGJ_2 e cancer*

O câncer e a inflamação estão relacionados pela epidemiologia, histopatologia, perfis inflamatórios e a eficiência de drogas anti-inflamatórias. Estas observações proporcionaram um impulso para a investigação e hipóteses sobre os mecanismos e a relação entre câncer e inflamação (RAKOFF-NAHOUM 2006).

Em um estudo realizado com 119 pacientes, demonstrou que a PGE_2 está aumentada e associada com a proliferação e invasão do câncer de endométrio (KE et al 2016) em outro trabalho, demonstrou que, a PGE_2 está envolvida no

desenvolvimento e proliferação na leucemia mieloide e câncer de mama (OBERMAYER et al 2012, BISHOP-BAILEY et al 2002), de outro modo a 15d-PGJ_2 uma prostaglandina com efeitos anti-inflamatórios, foi demonstrado que células do câncer de próstata foram tratadas com 15d-PGJ_2, diminuiram a expressão e sinalização dos receptores de andrógenos, que são essenciais para o desenvolvimento e progressão no câncer de próstata (KAIKKONEN et al. 2013)

O PPAR-γ está expresso em diversos tipos de câncer, (HERRERA et al 2015). A 15d-PGJ_2 e outros ligantes de PPAR-γ não prostanóides, como a classe dos glitazones, tem demonstrado em diversos experimentos *in vitro* e *in vivo*, elevada ativada antitumoral e antiangiogênico, inibindo consideravelmente o crescimento de células tumorais e induzindo apoptose em células de câncer de mama, cólon e pulmão (ALLRED et al. 2005; FROHLICH; WAHL, 2015; HORITA et al 2015).

Foi demonstrado que enquanto a 15d-PGJ_2 é capaz de inibir a atividade do NF-κB e a produção de óxido nítrico em células de cultura de micróglia, outro agonista de PPAR-γ o troglitazone não teve nenhum efeito, indicando que a 15d-PGJ_2 pode ser mais eficaz que outros agonistas de PPAR-γ (PETROVA, AKAMA, VAN-ELDIK, 1999) e com mecanismos independentes de PPAR-γ; (CHAWLA et al., 2001) alguns destes efeitos podem ser mediados através de interações covalentes entre a 15d-PGJ_2 e proteínas intracelulares conferindo melhores propriedades imunofarmacológicas se comparado aos glitazonas. Além do fator nuclear-κB (NF-κB) e a via de sinalização ERK também sido modulada pela 15d-PGJ_2 independente de PPAR (SCHER, PILLINGER, 2005).

1.3 Via da lipoxigenase

Uma vez liberado, o AA pode ser metabolizado pela via da lipoxigenase que tem como enzima chave a 5-Lipoxigenase (FUNK, 2001; HIKIJI et al., 2008), que a partir do AA promove a formação do leucotrieno A_4, que podem sofrer hidrolise enzimática e formar o leucotrieno B_4 (LTB_4) ou ser conjugado com glutamina dando origem ao leucotrieno C_4 e subsequentemente ao D_4 e E_4 (LTC_4, LTD_4 e LTE_4)

(HAEGGSTROM; WETTERHOLM, 2002). Os leucotrienos atuam como mediadores próinflamatórios onde o grupo LTC_4, LTD_4 e LTE_4 são produzidos predominantemente por basófilos, mastócitos e eosinófilos enquanto macrófagos e neutrófilos geram LTB_4 (FUNK; CHEN, 2000; FUNK, 2001). Inibidores da via da lipoxigenase tem demonstrado efeitos promissores na redução de mediadores proinflamatorios (WERZ; STEINHILBER, 2006).

1.4 Via do Citocromo P450 epoxIgenase

Os ácidos Epóxieicosatrienoicos (EETs) são produtos do metabolismo do ácido araquidônico, catalisados pelo citocromo P450 epoxigenase, os EETs possuem importantes atividades biológicas como vasodilatadores e propriedades anti-inflamatórias. Através da ação da enzima Epoxido Hidrolase solúvel (sEH) os EETs são convertidos em ácidos Dihidroxieicosatrienoicos (DHET), esta forma de diol, tem uma ação biológica bem reduzida, sendo assim a inibição da sEH tem sido proposto com uma abordagem para o tratamento da hipertensão e modulação das respostas inflamatórias (NORWOOD et al 2010).

A inflamação crônica é um fator importante que contribui para uma variedade de doenças humanas, incluindo artrite reumatoide, doença periodontal, psoríase e aterosclerose. Em condições fisiológicas o ácido araquidônico encontra-se esterificado nos fosfolipídios de membrana sendo mobilizados durante o processo inflamatório pela fosfolipase A2 que é ativada por estímulos químicos, mecânicos e produtos bacterianos, a epoxidação do ácido araquidônico por enzimas do citocromo P450 promovem a conversão para EETs, que tem uma variedade de efeitos biológicos, incluindo a modulação da inflamação (HUANG et al 2016).

Recentemente, foi demonstrado que a administração de EETs em ratas ovariectomizadas, impediu a perda óssea em modelo experimental de osteoporose, e inibiu a osteoclastogênese tanto *in vitro* como *in vivo*. Este resultado foi possível, pois o EETs inibiram múltiplas vias intracelulares após a ativação do RANK (Receptor ativador do fator nuclear k B), incluindo MAPKs (Proteíno-quinases ativadas por mitógenos), NF-kB, AP-1 (Ativator protein 1), PI3K, e ROS (Espécie reativa de oxigênio). Além disso, os EETs diminuíram os níveis séricos da razão

RANKL/OPG, TNF-α e IL-1β, que podem também ter contribuído para a atenuação da osteoclastogênese (GUAN et al., 2015).

No entanto, os EETs como descrito acima, tem uma meia vida curta o que dificulta seu uso farmacológico, porém, existem alguns inibidores da enzima sEH a fim de impedir a sua metabolização em diol e que poderiam ser utilizados como ferramenta farmacológica.

1.4.1 Epoxido Hidrolase solúvel (sEH)

Os níveis de EETs são regulados pela epóxido hidrolase solúvel (sEH), a principal enzima responsável pela sua degradação e conversão para um estado menos ativo, os ácidos dihidroxieicosatrienoicos (DHETs); limitando assim muitas das ações biológicas dos EETs. A sEH humana é codificada pelo gene EPHX-2 (SANDBERG; MEIJER, 1996) localizado no braço curto do cromossomo 8 (8 p-21-p12), com um comprimento aproximado de 45 kb, composto por 19 exons (LARSSON et al 1995), e nos mamíferos é uma enzima composta por dois monômeros de 62 kDa (CRONIN et al 2003; NEWMAN et al 2003). A sEH é composta por dois domínios, separados por um curto ligante, rico em prolina . O domínio N-terminal apresenta uma atividade de fosfatase que hidrolisa o fosfato de lípidos, enquanto o domínio C-terminal exibe uma atividade epóxido hidrolase que converte epóxidos para os seus dióis correspondentes. A inibição farmacológica e molecular da sEH tem sido estudada extensivamente para benefícios sobre o sistema cardiovascular. Estudos mais recentes sugerem a importância de EETs sob a inibição da sEH na dor e inflamação (BETTAIEB et al 2015).

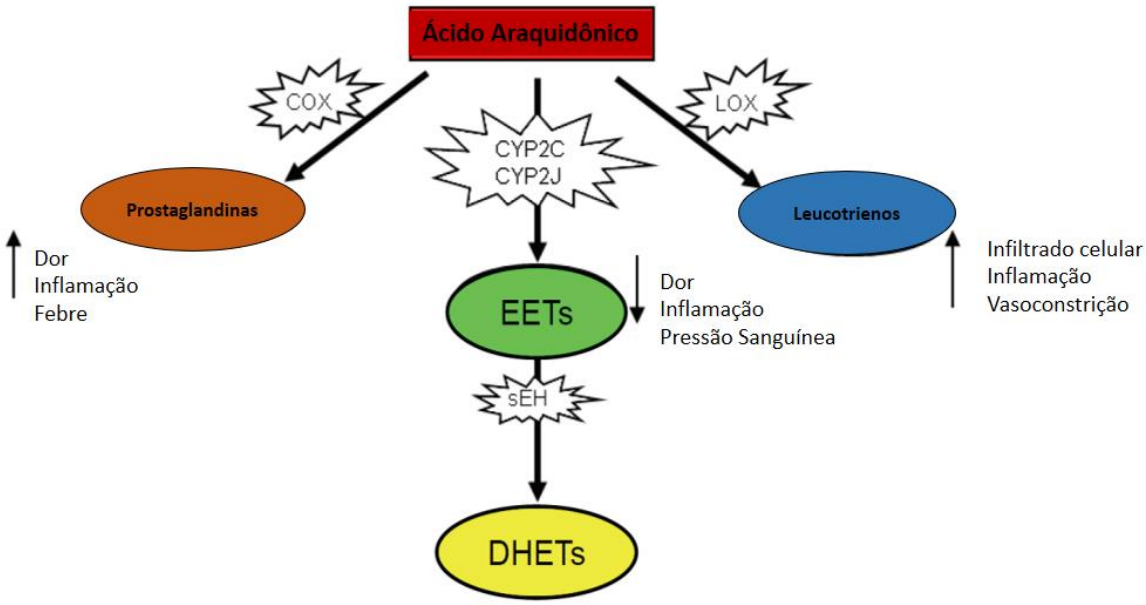

Figura 2: Metabolismo do ácido araquidônico e efeitos fisiológicos dos metabolitos do ácido araquidônico. COX=Ciclooxigenase, CYP2C=Citocromo P450 2C, CYP2J=Citocromo P450 2J, EETs=ácidos epóxi-eicosatrienóico, DHETs= dihidroxi-eicosatrienóico, sEH=epóxi hidrolase solúvel, LOX=Lipoxigenase. Adaptado e modificado de Nortwood et al 2010.

Kundu et al 2013, investigando a importância da quimiotaxia por MCP-1, descobriram que inibidores da ciclooxigenase (COX) não impediram a migração de monócitos tratados com MCP-1, entretanto no mesmo experimento curiosamente a migração de macrófagos foi diminuída ao inibirem de forma farmacológica o citocromo P450 e a sEH demonstrando que este efeito era dose dependente com uso de inibidores. Por outro lado quando foi adicionado os DHETs este processo de migração foi restaurado, demonstrando que os DHETs são importantes mediadores no processo de quimiotaxia mediado pelo MCP-1.

Em um trabalho utilizando um modelo de pancreatite aguda, BETTAEB et al 2015 demonstraram que os animais tratados com inibidor de sEH apresentaram uma redução na produção de citocinas proinflamatórias como TNF-α, IL-1β e IL-6, em outro trabalho foi demonstrado que animais geneticamente modificados para deficiência da sEH apresentaram uma redução sistêmica de citocinas como TNF-α, IL-1β e MCP1, quando comparados com animais não deficientes para sEH. (VANELLA et al 2015).

Estes dados demonstram a importância da inibição farmacológica da sEH, sendo assim está enzima se configura em um importante alvo para o tratamento e modulação das respostas inflamatórias.

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CAPÍTULO 2.

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 induces apoptosis and upregulates SOCS3 in human thyroid cancer cells

Carlos Antônio Trindade-da-Silva,[1,2,3] Carolina Fernandes Reis,[1,5] Lara Vecchi,[1] Marcelo Henrique Napimoga,[4] Marcelo Sperandio,[4] Bruna França Matias Colombo,[1] Patrícia Terra Alves,[1] Laura Sterian Ward,[5] Carlos Ueira Vieira,[2] Luiz Ricardo Goulart Filho.[1,6]

[1]Laboratory of Nanobiotechnology, Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil.
[2]Laboratory of Genetics, Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil.
[3]Hammock Laboratory of Pesticide Biotechnology, University of California Davis, 95616 Davis, CA, United States.
[4]Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, 13045-755 Campinas, SP, Brazil.
[5]Laboratory of Cancer Molecular Genetics, University of Campinas, 13081-970 Campinas, SP, Brazil.
[6]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA.

ABSTRACT

The cyclopentenone prostaglandin 15-deoxy-$^{\Delta(12,14)}$-prostaglandin J_2 (15d-PGJ_2) is a natural ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ) and a potential mediator of apoptosis in cancer cells. In the present study we evaluated the effect of 15d-PGJ_2 in human thyroid papillary carcinoma cells (TPC-1) using different doses of 15d-PGJ_2 (0.6 to 20 µM) to determine the IC_{50} (9.3 uM) via the MTT assay. The supernatant culture medium of the TPC-1 cells that was either treated with 15d-PGJ_2 or with the vehicle (control) for 24 hours was assessed for IL-6 secretion via CBA assay. RT-qPCR was used to evaluate mRNA expression of IL-6, SOCS1, SOCS3 and STAT3. TPC-1 cells treated with 15d-PGJ_2 decreased secretion and expression of IL-6 and STAT3, while increased SOCS1 and SOCS3. Overall, we demonstrated that 15d-PGJ_2 downregulated the IL-6 signaling pathway and led TCP-1 cells into apoptosis. In conclusion, 15d-PGJ_2 shows the potential to become a new therapeutic approach for thyroid tumors.

1. INTRODUCTION

Thyroid cancer combined with some of the commonest endocrine cancers make up the 5th commonest neoplastic disease in humans, which are increasing in incidence more rapidly than any other. The treatment of thyroid cancer consists mainly of surgical excision and ablation of the remaining tissue using radioactive iodine, which is only effective in non-metastatic primary tumors. Metastatic disease and recurrence are mostly incurable and require advanced therapeutic strategies to improve survival [1].

The molecular pathogenesis of thyroid cancer and several signaling pathways involve Signal Transducers and Activators of Transcription (STATs), which are a family of transcription factors that regulate cell proliferation, differentiation, apoptosis, immune and inflammatory responses, as well as angiogenesis [2,3]. Cumulative evidence has established that STAT3 plays a critical role on the development [4] and mediation of oncogenic signaling in many different cancers [5]. Phosphorylation of STAT3 can be induced via stimulation of the heterodimeric receptor complex by the IL-6 cytokine family, including IL-6, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, IL-11 and cardiotrophin-1 [6]. Moreover, STAT3 phosphorylation must be precisely controlled to maintain cellular homeostasis during both embryonic and adult development, requiring the participation of several negative regulators [7].

These negative regulators include cytoplasmic tyrosine phosphatases, e.g., protein tyrosine phosphatase 1B STAT, suppressor of cytokine signaling (SOCS) proteins, which block the cytokine receptor [8]. Loss of SOCS is known to contribute to abnormal activation of STAT3 in leukemia, lymphoma, hepatocellular carcinoma and non-small cell carcinoma of the lung [9].

Cyclopentenone prostaglandin 15-deoxy-$\Delta^{(12,14)}$ -prostaglandin J_2 (15d-PGJ_2), which is an endogenous molecule generated from the dehydration of PGD_2, is a natural ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ) and a potential mediator of apoptosis [10]. 15d-PGJ_2 has recently been demonstrated to exert both anti-inflammatory and anti-neoplastic effects in different cell lines and mouse models [11, 12, 13, 14, 15], although such effects have been shown to be largely independent from PPAR-γ [10], many of which are mediated via redox-modulating transcription factors, such as nuclear factor-kappaB (NF-kB), signal

transducers and activators of transcription 3 (STAT3), nuclear factor-erythroid 2p45 (NF-E2)-related factor (Nrf2), activator protein-1 (AP-1), hypoxia inducible factor, p53 and peroxiredoxins [16]. The electrophilic carbonyl group present in the 15d-PGJ2 cyclopentenone ring has been suggested as the main culprit for most such non-prostaglandin-like effects, since it promptly reacts with cysteine thiol groups of proteins that can be critical in the proliferative machinery of the cell [10].

Considering the cumulative evidence pointing towards a potent anti-neoplastic effect of 15d-PGJ_2 as well as the scarcity of studies investigating its effects on thyroid malignancies [17], the aim of this study was to evaluate the chemotherapeutic effect of 15d-PGJ_2 in thyroid cancer cells *in vitro*.

2. MATERIALS AND METHODS.

2.1 Cell line.

A papillary thyroid cancer (TPC-1) cell line was selected and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in humidified 5% CO_2 atmosphere at 37ºC. A normal fibroblast cell line (FG11) was cultured under the same conditions and used as control for cytotoxicity.

2.2 Analysis of cell viability

The effect of 15d-PGJ_2 on TCP-1 viability was evaluated using the MTT assay. Briefly, thyroid cancer cells were seeded in triplicates in 96-well plates containing 200 µL of DMEM + 10% FBS ($1x10^4$ cells per well) and incubated with 15d-PGJ_2 at concentrations ranging from 0.6 to 20 µM for 72 hours. Cells from each well were treated with 10 µL solution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) and plates were incubated for additional 4 h at 37°C. Sulfuric acid at 2N (200 µL/well) was added and mixed thoroughly to dissolve the dark-blue crystals. Absorbance of the converted dye was measured by spectrophotometry using a microplate reader at 570 nm (test) and 650 nm (reference). Cell survival was calculated as percentage of MTT inhibition as follows: % survival = (mean experimental absorbance/ mean control absorbance) × 100.

FG11 cells were also seeded as described above for TCP-1 cells. They were then incubated with 15d-PGJ_2 at concentrations ranging from 5 to 15 µM for 24, 48 and 72 hours. Cell count and viability were assessed on a Vi-Cell XR equipment (Beckman Coulter, USA).

2.3 Evaluation of apoptosis via annexin V staining

Drug-induced apoptosis was measured using annexin V-fluorescein isothiocyanate (Annexin V-FITC) and PI co-staining using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich). After 24 hours of treatment with 15d-PGJ_2, cells were rinsed and resuspended in 100 µL of staining solution (Annexin V-FITC and PI in HEPES buffer). Cells were then incubated at room temperature in the dark for 15 min, followed by the addition of 400 µL of binding buffer. The percentage of apoptotic cells was established by flow cytometry using a FACS Accuri C6 Flow (BD eBiosciences).

2.4 Cytokine Analysis

The effect of 15d-PGJ_2 on cytokines production by TPC-1 cells was evaluated in IMDM medium from 0 to 24 hours at 37ºC and 5% CO_2. This experiment was performed in triplicate using 24-well plates (1×10^4 cells/well). Cells suspensions were supplemented with 15d-PGJ_2 at 9.3 µM per well. Cytokines present in the culture supernatants were analyzed by BD Cytometric Bead Array (CBA) for Human Th1/Th2/Th17. This method uses beads with different fluorescence intensities in conjunction with a cytokine-specific capture antibody. Measurements were performed using the FL2 and FL3 channels of the Flow Cytometer Accuri C6 Flow (BD eBiosciences). A specific detection kit for IL-6 was used according to the manufacturer's protocols (BD eBioscience). Analysis output was obtained in the form of tables and charts using the FCAP Array™ Version 3.0 Software (BD eBioscience).

2.5 mRNA expression analyses

Quantitative real-time PCR (RT-qPCR) assays were performed using the Applied Biosystems 7500 Sequence Detecting system (Applied Biosystems, California, USA) and SYBRPremix Ex Taq II (Takara, Shiga, Japan) under the following reaction conditions: 40 cycles of PCR (95°C for 15 s and 60°C for 1 min) after an initial denaturation (95°C for 1 min). The primers used for amplification were as follows: SOCS3 - Forward: TCACCGAAAACACAGGTTCCA, and Reverse: GAGTATGTGGCTTTCCTATGCTGG; β-actin - Forward: CTACAATGAGCTGCGTGTGGC, and Reverse: CAGGTCCAGACGCAGGATGGC. Amplification of the housekeeping gene β-actin was used as an internal control to normalize the SOCS3 mRNA level. The RT-qPCR

data were presented as cycle threshold levels and were normalized against the corresponding β-actin control cycle threshold values. Relative gene expression was calculated using the $2^{(-\Delta\Delta C(T))}$ method, as described previously [18].

2.6 Statistical Analysis

The data were analyzed on GraphPad Prism (v.6.0c) software to compare the effects of different treatments. Two-way ANOVA and Bonferroni's *post-hoc* tests were used to analyze the data.

3. RESULTS

3.1 *In vitro* effect of 15d-PGJ_2 on TPC-1 and FG11 cell proliferation and viability.

15d-PGJ_2 decreased cell proliferation (Figs. 1 A and B) and cell viability at the concentrations of 10 µM and 20 µM (Fig. 1 C). These finding were used to calculate the IC50, which was established at 9.3 µM (Fig. 1D). This concentration was then used for subsequent experiments. The 15d-PGJ_2 did not show significant effect on fibroblast proliferation and viability in doses varying from 5 to 15µM (Fig. 2).

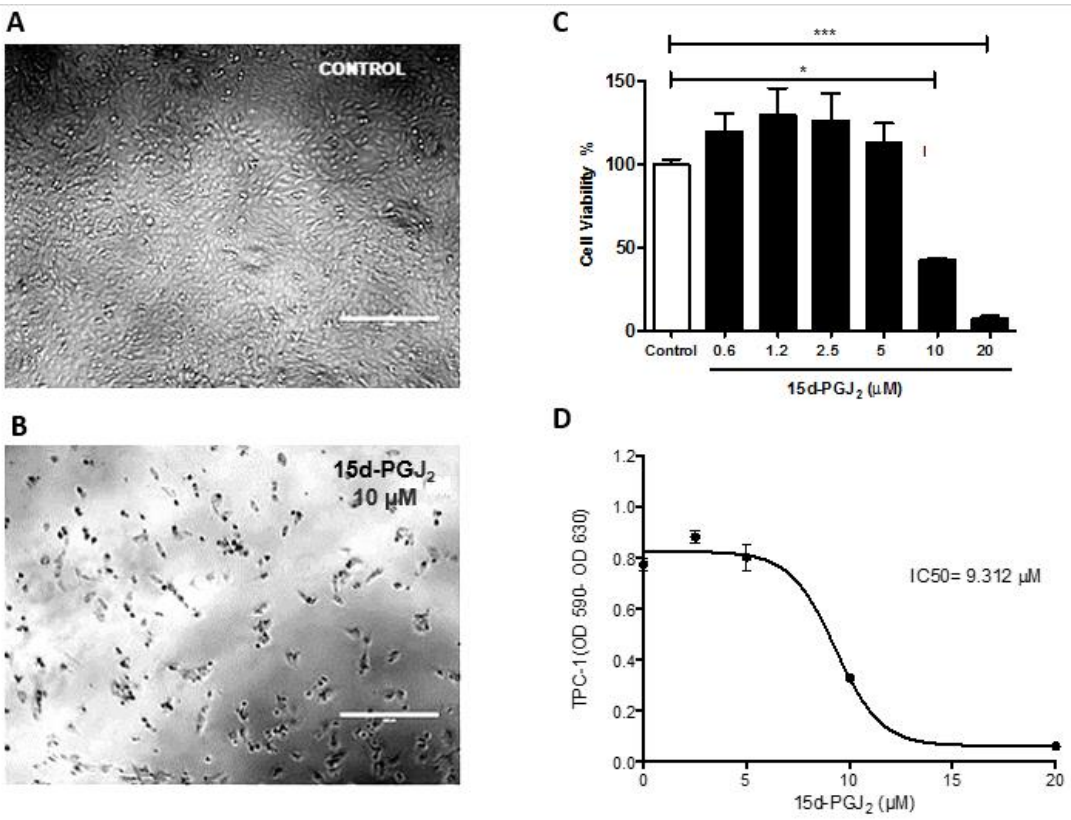

Figure 1. 15d-PGJ$_2$ decreased the viability of TPC1 cells. TPC1 cells were treated with 15d-PGJ$_2$, (A) represent the cell culture without treatment (B) cells treated with 10 µM of 15d-PGJ$_2$ (C) viability of the TPC1 cells treated with 15d-PGJ$_2$ in the concentrations of 0 to 20 µM (D) IC$_{50}$ from cell viability following treatment with 15d-PGJ$_2$. The data are presented as means + - standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control (* P> 0.01; *** p> 0.001).

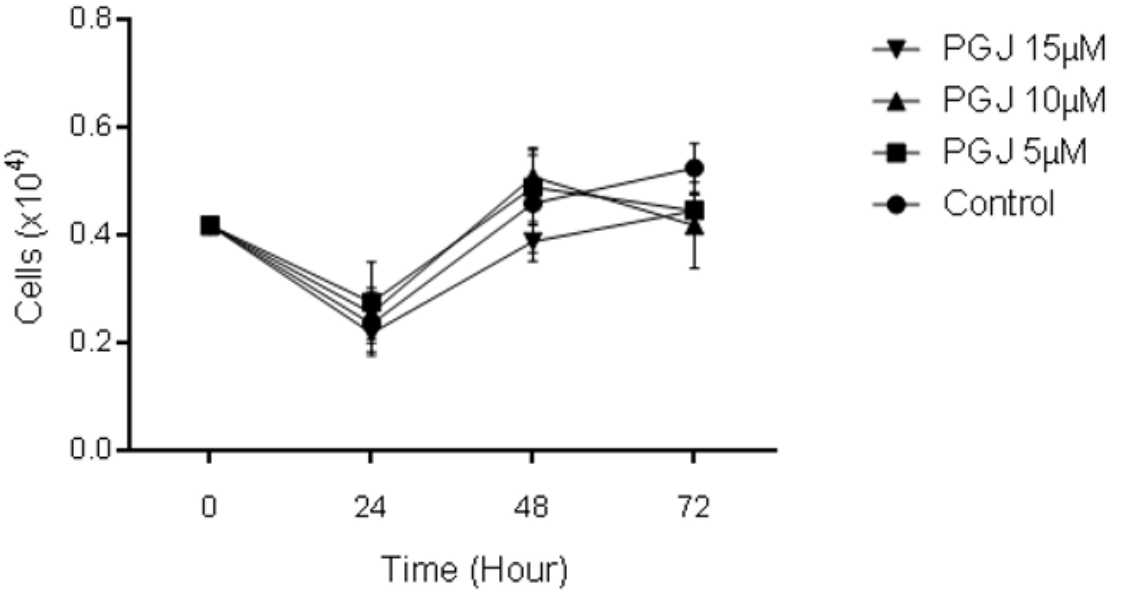

Figure 2. Fibroblast (FG11) cell proliferation under 15d-PGJ$_2$ treatment. FG11 cells were treated with 5 to 15 µM of 15d-PGJ$_2$. The data are presented as means ± standard deviation of three replicates from at least three independent tests. 15d-PGJ$_2$ did not show significant difference from the control at the doses of 5 µM, 10µM and 15µM.

3.2 Apoptotic effects of 15d-PGJ$_2$ on thyroid cancer cells

The annexin V apoptosis assay on TPC-1 showed that 47% of the cells treated with 15d-PGJ$_2$ (9.3 µM) entered apoptosis, whereas less than 5% was observed in the control group (Fig. 3).

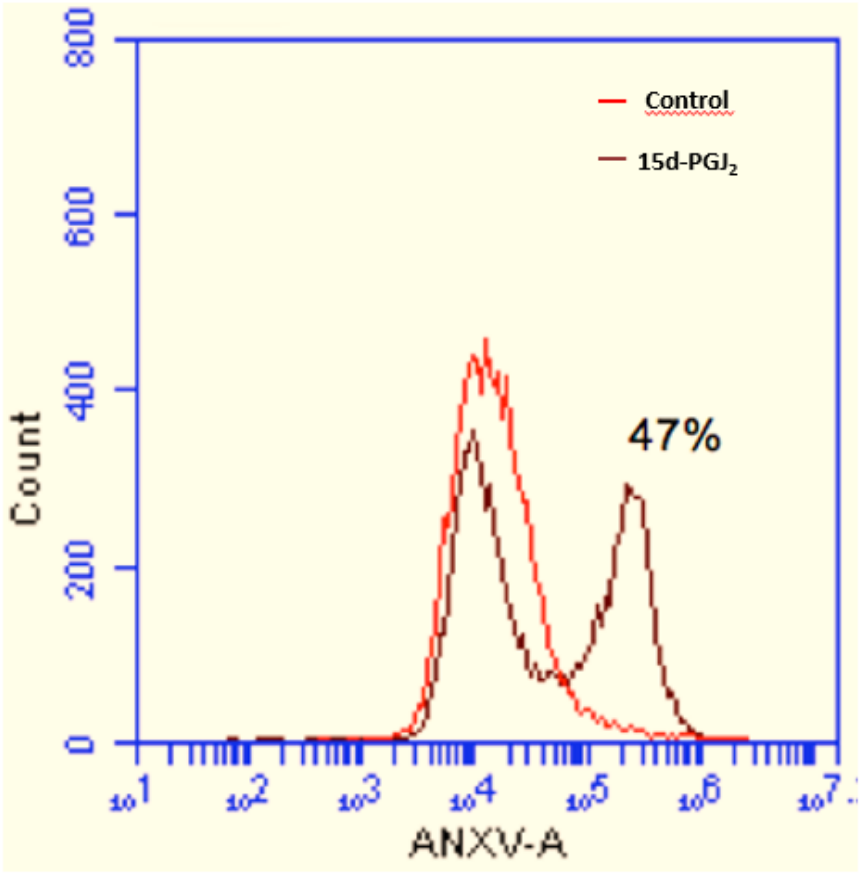

Figure 3. 15d-PGJ$_2$ induced apoptosis in TPC1 cells. The Annexin-V assay revealed that 15d-PGJ2 induced 47% apoptosis in TPC-1, compared to 5% in the control group. The data are presented as means + - standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control (*** p> 0.001).

3.3 Relative IL-6 mRNA expression and IL-6 release by TPC-1

The results revealed that IL-6 was highly expressed in TPC-1 and treatment with 15d-PGJ$_2$ decreased the relative IL-6 mRNA expression after 4 hours (Figure 4A). Concurrently, IL-6 release in the cell culture medium increased at a much lower rate than in the control group, thus demonstrating the down-modulation effect of 15d-PGJ$_2$ on IL-6 secretion by TPC-1 cells so soon as two hours after treatment (Figure 4B).

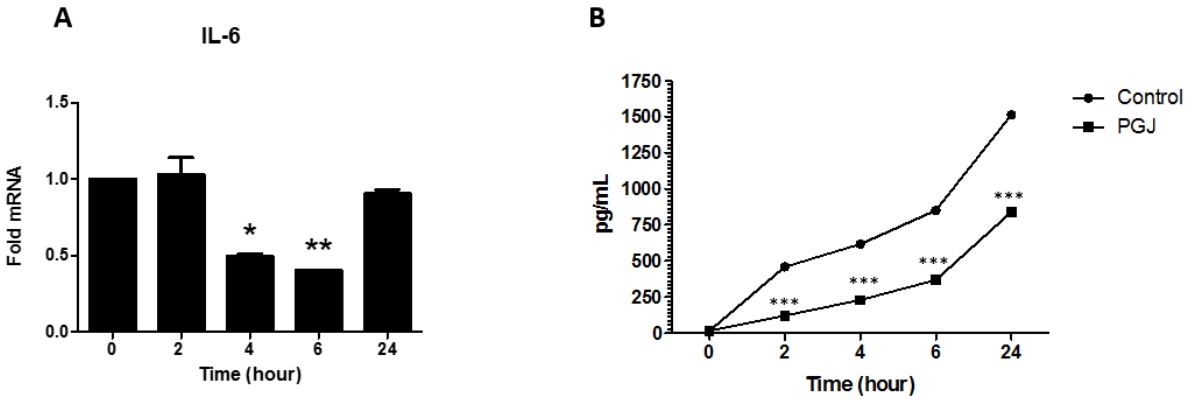

Figure 4: **decreased relative IL-6 mRNA expression and release - TPC1 cells treated with 15d-PGJ_2.** TPC1 cells were treated with 15d-PGJ_2 (9,8 uM) for 0 to 24 h (A) shows the relative IL-6 expression (B) quantitative IL-6 released by TPC-1 cells treated with 15d-PGJ2 against the control group. The data are presented as means + - standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control group (* P> 0.01; *** p> 0.001).

3.4 Relative expression of SOCS3, SOCS1 and STAT3

Upregulation of SOCS1 and SOCS3 occurred rather early in TPC-1 treated with 15d-PGJ_2 (Figure 5A and B). A significant difference between the control and the treated cells was observed two hours after treatment, with SOCS3 showing a four-fold increase in relative mRNA expression. Such an effect was not long-lasting, and 4 hours after treatment the expression of SOCS1 and SOCS3 was normalized. STAT3 was downregulated 4 hours after treatment and was maintained throughout the assay, for 24 hours (Figure 5C).

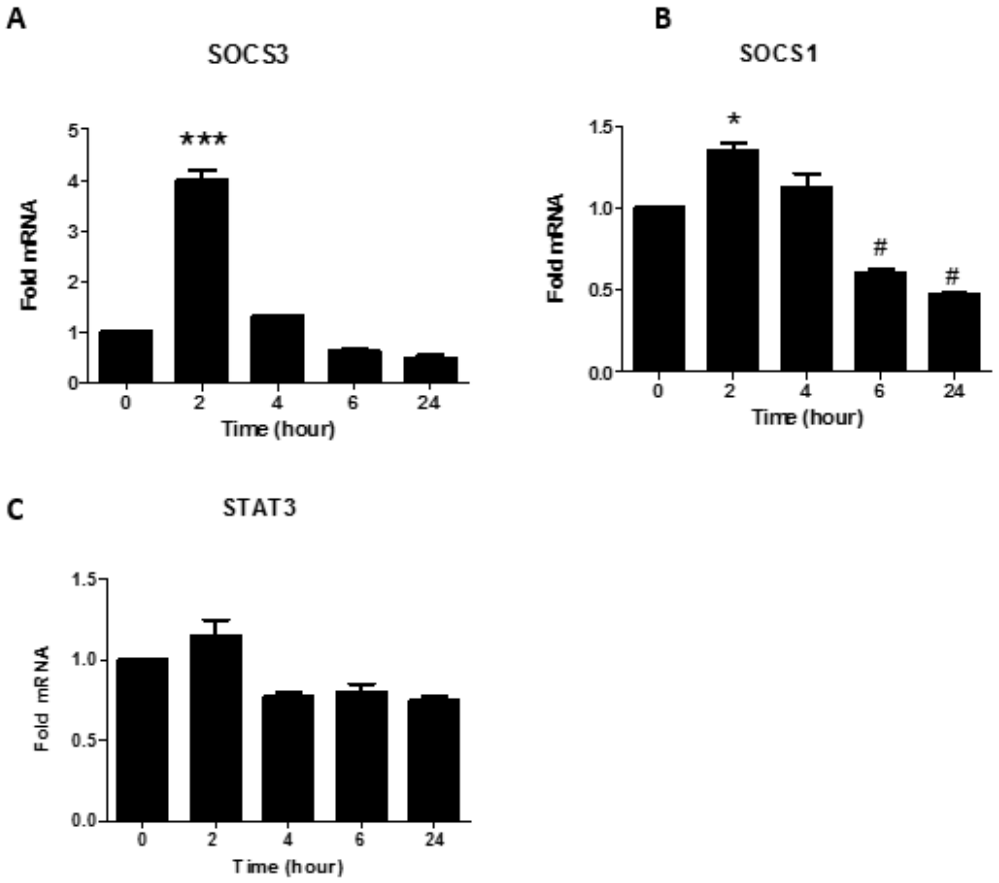

Figure 5: **SOCS 3 and SOCS1 increased in TPC-1 cells treated with 15d-PGJ2**. TPC1 cells were treated with 15d-PGJ_2 (9,8 uM) for 0 to 24 h (A) shows the relative expression of SOCS3 (B) SOCS 1 (C) and STAT3 (C) in the first two hours of treatment and decreased STAT3 four hours after the treatment (C). The date are presented as means + - standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control (* P> 0.01; *** p> 0.001).

4. DISCUSSION

The most important adverse aspects in the current surgical approach to treat papillary thyroid carcinoma is the risk of long-term recurrence and the difficulty in managing metastatic disease, especially in those cases initially regarded as low risk [19, 20] In the recent past, great efforts have been made to define new molecular therapies to potentiate the effectiveness of current cytostatic drugs and 15d-PGJ_2 has recently emerged as a potent antineoplastic molecule [21].

Several studies have demonstrated that although 15d-PGJ_2 is an endogenous ligand of PPAR-γ, most of its anti-neoplastic effects are PPAR-γ-independent [22, 23]. The effects of PPAR-γ ligands may also act by independent mechanisms because they differ widely amongst carcinoma types, and thus must be individually examined.

The present study investigated the role of exogenous 15d-PGJ_2 on papillary thyroid carcinoma cells, the TPC-1 cell line. The drug reduced cell viability at the doses of 10 and 20 µM (Figure 1C). Similar results have been found in cell viability in cultures with other cell lines of breast cancer, lung cancer, lymphoma [24, 25], colorectal [26, 27], ovarian [22], gastric [21], pancreatic [28] and prostate cancer [29].

Despite the overall anti-tumoral effect of 15d-PGJ_2, most studies have reported both dose and time-dependent responses, with lower doses often promoting opposing effects to the cytotoxic doses [23]. Micromolar doses of 15d-PGJ_2 are required to induce lymphoma cell death [30, 31], whereas physiological concentrations of the metabolite are in the range of picomolar to nanomolar [23, 32]. It has also been reported that high doses of 15d-PGJ_2 (≥5 µmol/L) caused cytotoxicity in cultured neurons, whereas low concentrations of the agonists (15d-PGJ_2, ≤1 µmol/L) suppress rat and human neuronal apoptosis and necrosis induced by H_2O_2 treatment [32].

Production of IL-6 and signaling are prerequisites for tumor progression [33]. Indeed, overproduction of IL-6 is commonly encountered in a variety of cancer cells and elevated serum IL-6 levels correlate with poor outcome in cancer patients [34, 35, 36]. IL-6 was shown to be an autocrine proliferation factor for tumor cell lines [37, 38, 39]. Additionally, STAT3 has been reported to be overexpressed in nearly 40% of all breast carcinomas due, in part, to autocrine expression of IL-6 [40]. In turn, paracrine IL-6 can induce autocrine IL-6 expression in cells within the tumor microenvironment, thus establishing an IL-6^+ niche and enhancing tumor progression [35]. The TPC-1 cells treated with 15d-PGJ_2 in the current study have shown a decrease in IL-6 expression and release associated with reduced cell proliferation, thus corroborating the aforementioned mechanism of IL-6-linked neoplastic progression in thyroid cancer cells. Recent studies have corroborated the inhibitory effect of 15d-PGJ_2 on IL-6 expression both *in vitro* [41] and *in vivo* [42].

Differently from normal cells, which phosphorylate STAT under stringent control, STAT3 is continuously phosphorylated in several neoplastic diseases via overproduction of agonists, such as specific cytokines, namely IL-6, and their respective cytokine receptors [40]. This cycle can be further enhanced via antagonism of negative regulators, such as SOCS and tyrosine phosphatases [43]. STAT3 has been reported to play an important role in maintaining cancer stem cells both *in vitro* and *in vivo*, implicating an integral involvement of STAT3 in tumor initiation, progression and maintenance [4]. In fact, this signaling route is so relevant in tumorigenesis that targeting STAT3 in neoplastic bone marrow disease practically interrupted the progression of metastasis [44, 45, 46, 47]. Cumulative evidence points to a clear STAT3-inhibitory effect of 15d-PGJ_2 in inflammatory diseases [48, 49, 10]. However, our findings show a small and stable decrease in the relative expression of STAT3 in thyroid cancer cells treated with 15d-PGJ_2 (Figure 5C), although not significant. It is possible that STAT3 phosphorylation was prevented by 15d-PGJ_2 through upregulation of SOCS3, which results in the inhibition of STAT3 activation, as shown elsewhere [50].

Upregulation of both SOCS3 and SOCS1 was also followed by downregulation of IL-6 expression in TCP-1 cells related to exposure to 15d-PGJ_2. SOCS3 is an inducible endogenous negative regulator of STAT3, and it is suggested as a tumor suppressor gene [51]. Negative modulation of SOCS1 and SOCS3 is a survival strategy in most cancer cells [52, 53, 54]. Conversely, overexpression of such cytokine inhibitors may indicate an anti-proliferative response. Indeed, our results have demonstrated that 15d-PGJ_2 increased SOCS3 on TPC-1 cells within two hours of contact with the drug, thus supporting the anti-oncogenic nature of this gene (Figure 5B). Interestingly, cells presented diminished levels of SOCS3 and SOCS1 six hours post treatment, which was extended to 24 hours post treatment (Figure 5 A and B), probably because 15d-PGJ_2 was already driving cells into apoptosis (Figure 3).

Regarding the downregulation of IL-6 mediated by SOCS3 overexpression, as early as two hours after exposure to 15d-PGJ_2, and considering the detrimental effects and actions of IL-6 linked with tumor growth, progression, and relapse [55, 56, 57], the 15d-PGJ_2 is presented as a novel anti-neoplastic drug.

Our data demonstrated that apoptosis was detectable in nearly 50% of the TPC-1 cells treated with 15d-PGJ_2, compared to 5% in the control group. We have also demonstrated that SOCS3 overexpression was an early event in treated cells, while STAT3 remained stable over 24 hours. It is known that activation of STAT3 in cancers leads to gene expression promoting cell proliferation and resistance to apoptosis [58], but 15d-PGJ_2-induced SOCS3 overexpression may have prevented STAT3 phosphorylation [50]. Despite the premature and short-lasting effect of 15d-PGJ_2 on SOCS3, its expressive upregulation (Figure 5A) may have been high enough to mediate apoptotic signaling within cells [59].

5. CONCLUSION

The present study shows important anti-proliferative and apoptotic activities in human thyroid cancer cells induced by 15d-PGJ_2, Such events are linked with overexpression of SOCS3 that inhibits IL-6 signaling, a key factor in many cancers. This is the first report on 15d-PGJ_2-induced SOCS3 expression, which evidences a novel therapeutic option for the treatment of thyroid cancer, and other cancers that are dependent on IL-6 signaling.

Conflict of Interests

The authors declare no conflict of interests.

Authors´ Contribution

Carlos Antonio Trindade da Silva and Carolina Fernandes Reis have equally contributed to this work.

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CAPÍTULO 3.

Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, RANK-related signaling, ER stress and apoptosis

Carlos Antonio Trindade-da-Silva[1,2,3†], Ahmed Bettaieb[4†], Marcelo Henrique Napimoga[3], Kin Sing Stephen Lee[1], Bora Inceoglu[1], Carlos Ueira-Vieira[2], Donald Bruun[6], Sumanta Kumar Goswami[1], Fawaz G. Haj[5] and Bruce D. Hammock[1].

† **Contributed equally to this work.**

1 Department of Entomology and Nematology and UC Davis Comprehensive Cancer Center, University of California, Davis, CA 95616, United States;
2 Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, 38400-902, Brazil;
3 Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas, 13045-755, Brazil;
4 Department of Nutrition, University of Tennessee-Knoxville, Knoxville, TN 37996
5 Nutrition Department, University of California, Davis, CA 95616;
6 Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616, United States.

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Running Title: Inhibition of sEH blocks alveolar bone loss

Corresponding author:

Bruce D. Hammock

Professor

Department of Entomology and Nematology & UCD Comprehensive Cancer Center, Director,

NIEHS-UCD Superfund Research Program PI, NIH Biotechnology Training Program

University of California – Davis

One Shields Ave, Davis, CA 95616, USA

(530) 752-7519 office

(530) 752-8465

message (530)

752-1537 fax

bdhammock@ucd

avis.edu

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Abstract
Epoxyeicosatrienoic acids (EETs), metabolites of arachidonic acid derived from the cytochrome P450 (CYP450) enzymes, are mainly metabolized by soluble epoxide hydrolase (sEH) to their corresponding diols. EETs but not their diols, have anti-inflammatory properties and inhibition of sEH might provide protective effects against inflammatory bone loss. Thus, in the present study, we tested the selective sEH inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) in a mouse model of periodontitis induced by infection with *A. actinomycetemcomitans*. Oral treatment of wild type mice with TPPU and sEH knockout (KO) animals showed reduced bone loss induced by *A. actinomycetemcomitans*. This was associated with decreased expression of key osteoclastogenic molecules RANK/RANKL/OPG and the chemokine MCP-1 in the gingival tissue without affecting bacterial counts. In addition, downstream kinases p38 and JNK known to be activated in response to inflammatory signals were abrogated after TPPU treatment or in sEH KO mice. Moreover, endoplasmic reticulum stress was elevated in periodontal disease but was abrogated after TPPU treatment and in sEH knockout mice. Together, these results demonstrated that sEH pharmacological inhibition may be of therapeutic value in periodontitis.

Key words: Periodontitis, inflammation, bone, epoxyeicosatrienoic acid, soluble epoxide hydrolase, TPPU.

Introduction

Periodontitis is a chronic inflammatory disease of the supporting tissues of the teeth with progressive attachment loss and bone destruction (Flemmig, 1999). Dysbiosis is thought to be one of the major drivers of this condition. Imbalance in the periodontal microbiota, more specifically the relative abundance of individual species of bacteria was hypothesized to affect the host–microbe interactions, ultimately leading to destructive inflammation and bone loss (Hajishengallis et al., 2012). However, more recent findings bring up the possibility that overgrowth of bacteria may be a resulting outcome rather than the cause of periodontitis. Thus, it is likely that the host response to bacteria that leads to the tissue changes noted in gingivitis and periodontitis is the root cause (Bartold and Van Dyke, 2013).

In chronic osteolytic inflammatory diseases such as periodontitis, the failure of endogenous resolution pathways seem to lead to tissue destruction and conversely, augmentation of these natural defensive mechanisms can be an effective approach to control these diseases (Van Dyke, 2011). Resolution of inflammation is now widely accepted as an active process, in which endogenous antiinflammatory and pro-resolving mechanisms actively mediate wound healing and tissue regeneration as opposed to fibrosis and scarring (Levy et al., 2012; Ortega-Gomez et al., 2013). More recent studies provide supporting evidence for this concept, where endogenous control of inflammation directly improves bone healing and regeneration and suppresses the flow of destructive inflammatory infiltrate into the tissue (Hasturk et al., 2007; Napimoga et al., 2012).

Arachidonic acid (ARA) is an omega-6 polyunsaturated fatty acid constituting the phospholipid domain of most cell membranes. It is released by phospholipases such

as cytoplasmic PLA2 and is metabolized into eicosanoids through three main routes, via prostaglandin-endoperoxide synthase/cyclooxygenases (PTGS/COX), lipoxygenases (LOX), and via the cytochrome P450s (CYP), resulting in prostanoids leukotrienes and hydroxy-eicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids (EETs). The CYP enzymes that utilize ARA as a substrate mainly produce EETs and the ω-hydroxyl metabolites 19- and 20-HETE. ARA is metabolized to four biologically active EET regioisomers, the 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. All EETs are then further metabolized into less active dihydroxy-eicosatrienoic acids (DHETs) by the enzyme soluble epoxide hydrolase (sEH, EC 3.3.2.10)(Morisseau and Hammock, 2013).

Existing drugs target the cyclooxygenase (COX) and lipoxygenase (LOX) branches of the ARA cascade (Marnett, 2009; Haeggstrom et al., 2010). These branches largely synthesize proinflammatory mediators such as the prostanoids and leukotrienes. Most recently discovered branch, the cytochrome P450 branch, however, has not been adequately exploited as a pharmaceutical target. This branch produces both anti- and to lesser degree pro-inflammatory metabolites, including the 19- and 20- hydroxy-eicosatetraenoic acids (19- and 20-HETE) (Williams et al., 2010). Notably, antiinflammatory epoxy fatty acids such as EETs, and EDPs (EpDPEs) are produced by cytochrome P450s (Spector, 2009; Imig, 2012). EETs seem to promote the resolution of inflammation, rather than prevent, in a manner similar to that exhibited by mediators in the LOX pathway (Serhan et al., 2011). EETs reduce inflammation, but are also analgesic, anti-fibrotic and anti-hypertensive, acting in both paracrine and autocrine fashion (Spector, 2009; Bettaieb et al., 2015; Harris et al., 2015). However, the *in vivo* instability of EETs because of their rapid metabolism by sEH impeded understanding the roles of these

lipid mediators until the development of potent and orally available sEH pharmacological inhibitors became available (Chacos et al., 1983; Morisseau et al., 1999).

The hydrolysis of EETs to DHETs by sEH was considered as an inactivation process in which bioactive metabolites were degraded to inactive products (Schmelzer et al., 2005). However, recent findings suggest that DHETs are also bioactive and are pro-inflammatory (Norwood et al., 2010), as are the diols linoleate epoxides (Viswanathan et al., 2003). Thus, sEH inhibition might be a powerful approach for reducing inflammation not only by stabilizing the anti-inflammatory mediators, but also by reducing pro-inflammatory mediator production. Furthermore, sEH inhibitors act in synergy with existing anti-inflammatory drugs including COX and LOX inhibitors(Schmelzer et al., 2006; Liu et al., 2010), as well as anti-inflammatory phosphodiesterase (PDE) inhibitors (Inceoglu et al., 2011).

The current study aimed to test the potential utility of sEH pharmacological inhibition in periodontitis, in which we evaluated the effects of sEH inhibition and EETs on bone loss using a mouse model of bacterial periodontitis.

Materials and Methods

Aggregatibacter actinomycetemcomitans (JP2) was purchased from ATCC (Manassas, VA) and cultured in-house under anaerobic conditions to ensure viability and virulance.

Chemicals: The sEH inhibitor 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl]urea was synthesized in-house, purified and chemically characterized as described earlier (Rose et al., 2010). The methyl ester of arachidonic acid was from NuChek Prep Inc (Elysian, MN). Chromatography grade solvents for HPLC were obtained from Fisher Scientific (Pittsburgh, PA). Standards

for LCMS/MS analysis were purchased from Cayman Chemical (Ann Arbor, MI). The EET methyl esters were synthesized, purified and characterized in-house using procedures published previously (Morisseau et al., 2010). The final regioisomeric mixture was analyzed using LC-MS/MS to ensure purity and regioisomeric ratio which was 2.2:1.6:1.1: 1, for 14, 15-:11, 12-: 8, 9-: 5, 6- EpETrE, respectively).

Bacterial viability: The potential bacteriostatic or bactericidal effects of TPPU at the administered dose were tested *in vitro*. The microbial inoculum of *A. actinomycetemcomitans* was prepared and adjusted to 5×10^6 colony forming units (CFU)/mL in tryptic soy broth. TPPU was then dissolved to a final concentration of 10 μM in polyethylene glycol (PEG400; Fisher Scientific, Nidderau, Germany) and was added and incubated at 37°C for 24 hours anaerobically. PEG400 solution without the inhibitor was used as a control. Experiments were performed in duplicates on three different days.

Animals and Animal Care: C57BL/6 (WT, wild type) and sEH -/- (KO, knockout) male mice were maintained under standard conditions, 23 ± 1˚C, 12-h light–dark cycle, *ad libitum* food and water in housing facilities at UC Davis. Animals were age-matched and each group consisted of 8 mice, 6–7 week old and weighing 20-25 g. All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were performed according to institutional guidelines for animal experimentation and were approved by the Animal Resource Services of the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care.

Periodontitis model and treatments: Animals orally received $1x10^9$ CFU/ml of a diluted culture of freshly grown *A. actinomycetemcomitans* JP2, in a volume of 100 µl PBS containing 2% carboxymethylcellulose. The solution was placed into the oral cavity with a micropipette, and the procedure was repeated at 48 and 96 h after the first inoculation. Treatment with TPPU and EETs was initiated after the third inoculation of bacteria. The inhibitor was dissolved in PEG400 and administered at 1mg/kg/day by oral gavage. For the treatment with EETs, we attempted to apply viscous solution of mixture of EET-methyl ester regioisomers dissolved in PEG400 to the gum tissue using a fine pipette at a dose of 1 µg/kg/day. The mice received the entire EET dose. This was done immediately before the oral gavage with both groups of mice receiving TPPU or mice receiving vehicle alone. Because EETs are unstable in stomach acid we assume exposure is largely topical or buccal. All treatments continued for 15 days. The plasma and blood concentration of TPPU was quantified as previously described by LC–MS analysis (Ostermann et al., 2015). The negative control group consisted of uninfected mice (sham-infected), the positive control group was infected and received the vehicle only. One day after the treatment period (16^{th} day), animals were sacrificed by cardiac puncture after anesthesia with xylazine/ketamine. Plasma and whole blood were sampled and frozen until analysis.

Quantification of alveolar bone loss: Evaluation of alveolar bone loss was performed as described previously (Napimoga et al., 2013). Sixteen days after the third inoculation, animals were sacrificed, the jaws were removed, and defleshed, then immersed overnight in 3% hydrogen peroxide, and stained with 1% methylene blue in PBS. Horizontal bone loss was assessed morphometrically by measuring the distance between the cement–enamel junction and the alveolar bone crest of the

first and second molars. Measurements at 14 buccal sites per mouse (7 sites each on the left and right maxillary molars) were made under a microscope, pictures were taken and bone measurements were analyzed using the Image J software suit. Random and blinded measurements were taken by the same calibrated person (C.A.T.d.S.). Intraexaminer reproducibility of the measurements achieved >90%.

Western Blotting: Western blotting was performed as described earlier (Bettaieb et al., 2013). Briefly, tissues were lysed first and clarified by centrifugation and protein concentrations were determined using the bicinchoninic acid protein assay kit (ThermoFisher Scientific, Waltham MA). Equal amounts of protein (20 µg) from the gingival tissue were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblotting of lysates was performed with antibodies for MCP1 (Biolegend; San Diego, CA), pp38 (Thr180/Tyr182), p38, pJNK (Thr183/Tyr185), JNK or cCasp3 (Cell Signaling Technology; Beverly, MA), RANK, RANKL, OPG or α-Tubulin (Santa Cruz Biotechnology; Santa Cruz, CA). Antibodies for sEH were generated in-house using recombinantly expressed mouse sEH following affinity purification. After incubation with the appropriate secondary antibodies, proteins were visualized using Luminata™ Fort Western HRP substrate (Millipore). Pixel intensities of immunoreactive bands were quantitated using FluorChem Q Imaging software (Alpha Innotech). For phosphorylated proteins data were presented as phosphorylation level normalized to total protein expression and for non-phosphorylated proteins as total protein expression normalized to α-Tubulin.

Statistical analysis: The statistical analyses were performed using Prism 5.0 or the SigmaPlot Software Suit. The data were first examined for normality using the Kolmogorov-Smirnov test, and then analyzed using one-way ANOVA. If there was a significant among-subjects main effect for the treatment group following one-way

ANOVA, or one way ANOVA on ranks, post-hoc contrasts, using the Student Newman Keuls multiple comparison or Tukey's all pairwise post-hoc tests. Data are presented as mean ± S.E.M.

Results

TPPU does not affect bacterial viability

In the present study we tested the potential effects of TPPU, a potent and selective pharmacological inhibitor of sEH, to inhibit bone loss caused by periodontal disease in mouseinduced periodontitis model. TPPU or its vehicle did not have bacteriostatic or bactericidal effect on the cultures used to induce the periodontal disease. As demonstrated in Supplemental Figure 1, no change in the growth of the *A. actinomycetemcomitans* was observed in the presence or absence of TPPU.

Chemical inhibition of sEH reduces bone loss

There were no *A. actinomycetemcomitans* in the oral cavities of mice prior to deliberate infection. On the other hand, persistent oral colonization by the pathogen was confirmed in all infected animals on the last day. Next, bone loss was quantified in all mice (Fig. 1A). Sham-infected animals presented the lowest distance between CEJ (cement-enamel junction) and ABC (alveolar bone crest) during the experimental period (Figure 1B). On the other hand, animals orally infected with *A. actinomycetemcomitans*, but received no treatment, showed significantly greater bone loss when compared to the uninfected animals (Figure 1C). Animals infected and orally treated with

Figure 1.

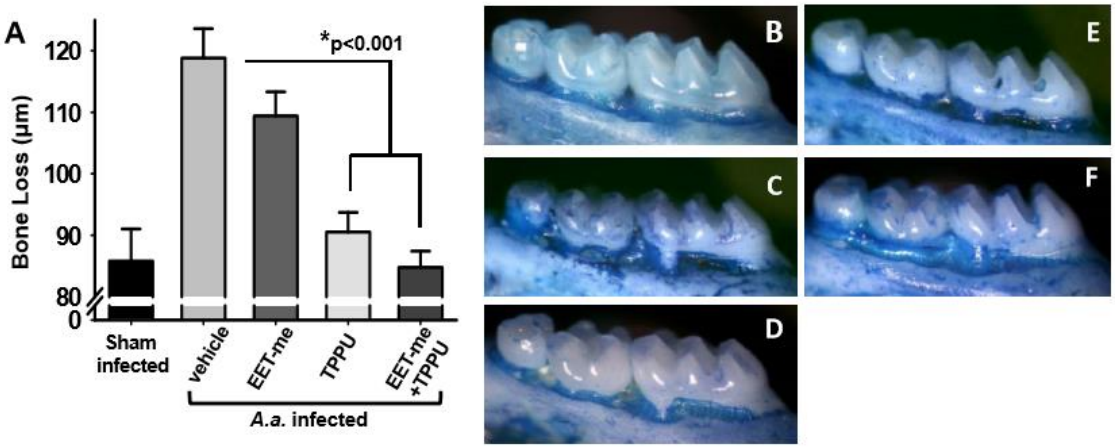

Figure 1: Inhibition of sEH with a potent and orally available small molecule TPPU decreases bone loss. Mice were inoculated with freshly grown cultures of *A. actinomycetemcomitans* on three consecutive days as described in the methods. The vehicle PEG400, TPPU (1mg/kg) and EETmethyl esters (1 µg/kg diluted in PEG400) were orally administered daily by gavage. Treatments continued until 15th day post infection and samples were obtained on the 16th day. Distance (µm) between the cement-enamel junction and the alveolar bone crest for all experimental groups were quantified. (A) The group vehicle alone developed significant bone loss, whereas groups treated with TPPU and TPPU+EET-methyl esters displayed a marked reduction in bone loss. Mice receiving EET-methyl esters were not different than vehicle treated infected mice, suggesting the necessity of inhibiting sEH for EETs to display activity. (B-F) Panels display deboned and methylene blue stained teeth from sham (B, n=8), mice infected with *A. actinomycetemcomitans* (C, n=14), EETmethyl ester (1 µg/kg/daily) treated (D, n=12), TPPU,1 mg/kg treated (E, n=13), and EET-methyl ester +TPPU treated groups (F, n=13). The dark stained areas indicate sites of bone loss. The results are expressed as mean ± SEM (*p < 0.001, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

EET-methyl esters at a dose of 1 µg/kg/day did not display a significant reduction in bone loss (Figure 1D). In contrast, mice treated with TPPU had significantly lower bone loss than the untreated infected animals and was comparable to the levels of uninfected animals (Figure 1E). Moreover, consistent with this observation the mice that received combination of TPPU and EET-methyl esters had significantly lower bone loss compared to untreated infected animals (Figure 1F). However, this decrease was not significantly more than the TPPU group (p=0.53). Using this dosing scheme, one would expect a near complete and sustained inhibition of sEH

activity. Blood concentration of TPPU at the end of the experiment also supports the argument that the target enzyme is significantly inhibited. Groups of mice that received TPPU and TPPU+EET-methyl esters had more than 8 µg/mL of TPPU detected in the blood, even after 24 h of the last oral administration (Supplemental Figure 2). There were no differences regarding the blood TPPU concentration in the group that received TPPU+EET-methyl esters. These levels reiterate the argument that the target enzyme is significantly inhibited (Liu et al., 2009; Liu et al., 2010; Rose et al., 2010). Thus, in these studies TPPU was sufficiently stable *in vivo* to provide effective concentrations throughout the course of the experiment.

Genetic ablation of sEH recapitulates the effects of sEH inhibitor on bone loss

To support the results of sEH pharmacological inhibition, we performed similar experiment using sEH global knockout mice. Consistent with the results obtained earlier, wild type mice infected with *A. actinomycetemcomitans*, showed significantly greater bone loss when compared to the uninfected animals (Figure 2). Remarkably, infected sEH knockout mice displayed highly significant reduction in bone loss, similar to the levels of uninfected group (Figure 2D and 2E). Findings using sEH knockout mice recapitulate observations from sEH pharmacological inhibition using TPPU.

Figure 2.

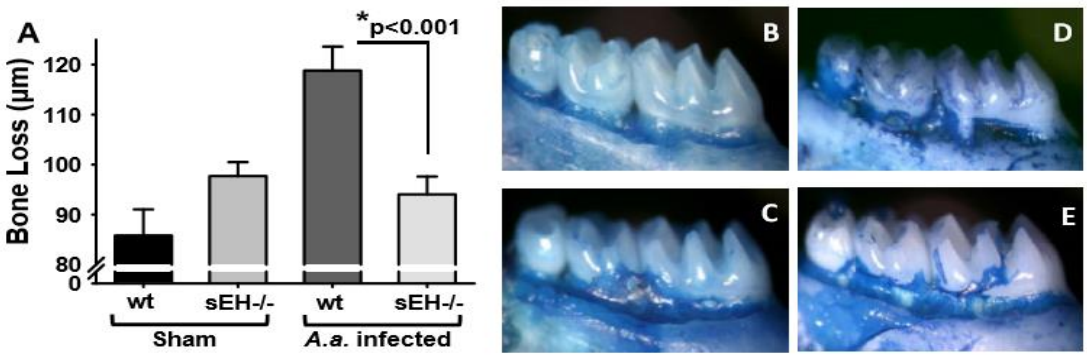

Figure 2: Genetic inhibition of sEH by gene knockout decreases bone loss similar to chemical inhibitor. sEH$^{-/-}$ and wild type C57/B6 mice were from a UC Davis maintained colony. Mice at age 6 weeks were infected with *A. actinomycetemcomitans* three consecutive times as described for Figure 1 and at the end of the treatment period distance (µm) between the cement-enamel junction and the alveolar bone crest for all experimental groups were quantified. In parallel to the results with the sEH inhibitor TPPU the genetic knockout of sEH resulted in significantly reduced bone loss. Panels display wild type sham infected (B, n=8)), sEH$^{-/-}$ sham infected (C, n=15), wild type mice orally infected with *A. actinomycetemcomitans* (D, n=14), and sEH$^{-/-}$ mice, orally infected with *A. actinomycetemcomitans* (E, n=14). The dark stained areas indicate sites of bone loss. The results are expressed as mean ± SEM (*p < 0.001, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

Inhibition of sEH alters key regulators of bone remodeling

To understand the basis of these effects we determined the levels of key regulators of bone remodeling, a highly dynamic process. The RANK/RANKL/OPG system is generally accepted as a master regulator of bone loss and regeneration (Lacey et al., 1998). RANKL synthesized by osteoblasts, cells that synthesize new bone, targets RANK on the osteoclasts, cells that resorb bone.

This stimulates bone loss by activating osteoclasts. The third arm of the system is osteoblast derived OPG, which is a soluble decoy receptor for RANKL and prevents its binding to RANK and thereby fine tuning bone remodeling. As expected, in infected control mice, levels of RANK, RANKL, OPG and MCP-1 in the gum tissue were increased, compared to uninfected animals (Figure 3). This reiterates the imbalance in bone remodeling process as well as increased infiltration of cells that mediate inflammation, arguments supported by data presented here. In contrast, TPPU treatment significantly reduced the expression of these biomarkers. Consistent with the TPPU group, animals treated with TPPU+EET-methyl esters (1 µg/kg) displayed a nearly identical profile in the expression of all four proteins quantified (Figure 3). Equally importantly, in sEH KO mice infected with *A. actinomycetemcomitans*, the effects of TPPU treatment were recapitulated. More specifically, in sEH KO mice levels of RANK, RANKL, and MCP-1 were similar to TPPU treated group, while they displayed slightly higher levels of OPG (Figure 3B-E). However, the higher levels of OPG could be seen as an advantage since this would lead to less bone loss. Overall, these results underline the accelerated and incongruent bone remodeling in periodontal disease and that these pathological changes can be re-calibrated by sEH deletion or pharmacological inhibition.

Figure 3.

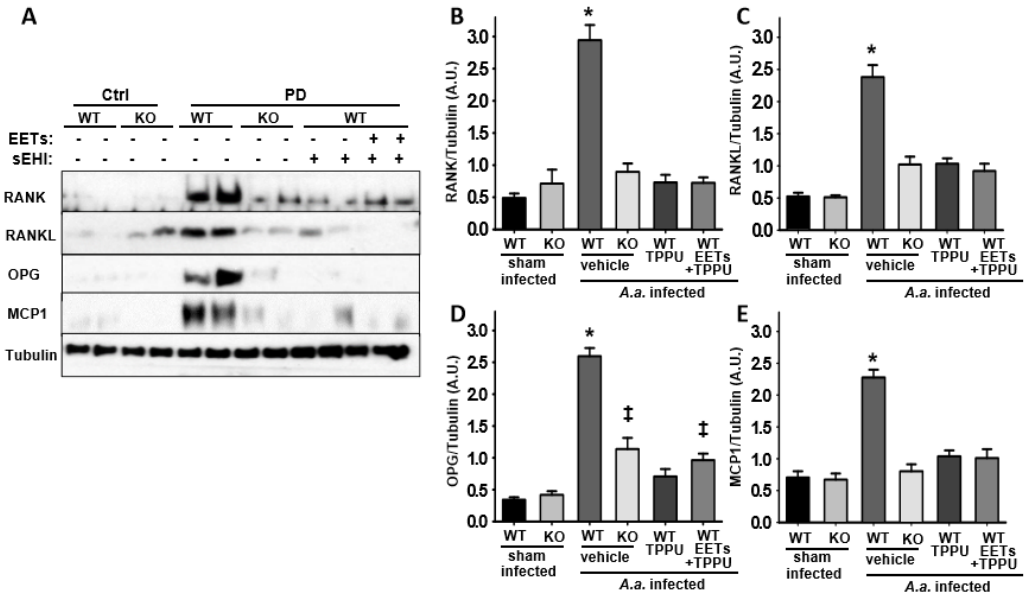

Figure 3: The dysregulated RANK/RANKL/OPG system in periodontal disease is restored by chemical or genetic ablation of sEH. Protein expression levels of osteoclastogenesis-related factors in gingival tissues from all experimental groups were investigated by Western blotting. For quantification, band intensity was normalized to that of α-tubulin. Protein band intensity is represented as arbitrary units. Density quantification included all animals and mean± SEM of each group (n=6 per group) are displayed in the bar graphs. (A) Original blots displaying two randomly selected animals. (B) Bar graphs of mean band intensity for, RANK (B), RANKL (C), OPG (D) and MCP-1(E) measured for all six mice. (*$p < 0.001$, ‡ $p<0.03$, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

The significant reduction in MCP-1 expression is suggestive of a decrease in inflammatory cell migration and therefore inflammation. Therefore, we further monitored key downstream kinases known to be phosphorylated in response to inflammatory signals. Two stress kinases, p38 and JNK activate their respective signaling cascades, increase inflammation, cytokine synthesis and apoptosis.

Figure 4.

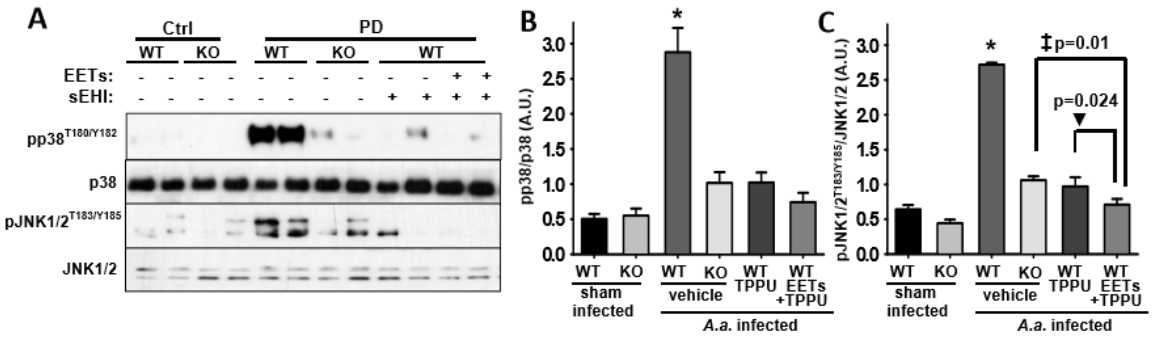

Figure 4: Periodontal disease mediated phosphorylation of pro-inflammatory p38 and JNK1/2 is reduced by chemical or genetic ablation of sEH. Phosphorylation and activation of p38 and JNK1/2 were quantified from all groups by normalizing band intensity to that of α-tubulin. (A) Original blots displaying two randomly selected animals. (B and C) Bar graphs of phosphorylation status of p38 and JNK1/2. Mean band intensity is measured for all six mice and are represented as arbitrary units (mean ± SEM). (*p < 0.001, ‡ p=0.01, ▼p=0.024, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

In the gingival tissue of infected mice, levels of pJNK (phosphorylated JNK) and pp38 (phosphorylated p38) were largely increased supporting the idea that dysregulated host responses have a pivotal role in periodontal disease. In contrast, treatment with TPPU, TPPU+EET-methyl esters or in sEH KO mice infected with *A. actinomycetemcomitans*, phosphorylation of both kinases were greatly reduced (Figure 4). These observations are consistent with the argument that sEH inhibition

dampens inflammatory response in periodontal disease.

Figure 5.

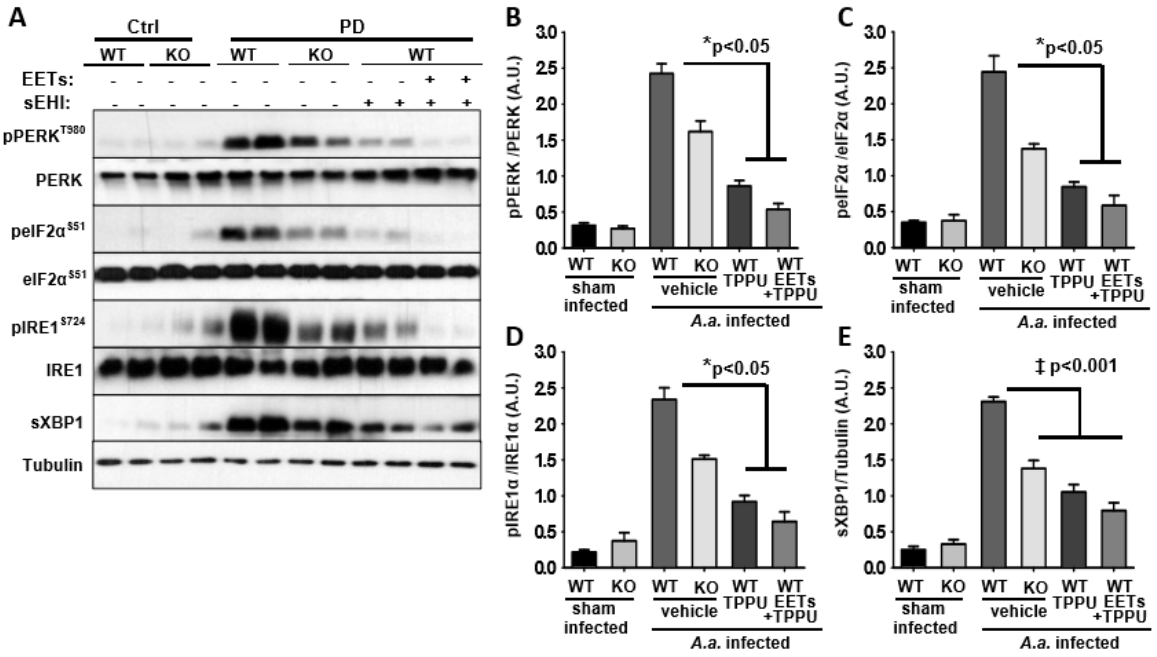

Figure 5: ER stress sensors are activated in gingival tissues of mice with periodontal disease and reversed by inhibition of sEH (A) Original blots displaying two randomly selected animals for each group. (B to E) Bar graphs of phosphorylation status of PERK, eIF2α, IRE-1α and expression level of sXBP-1 normalized to expression of α-tubulin. Mean band intensity is measured for all six mice and are represented as arbitrary units (mean ± SEM). (‡ $p < 0.001$, *$p<0.05$, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey's all pairwise multiple comparison post-hoc test).

The sEH is a significant regulator of endoplasmic reticulum (ER) stress response (Bettaieb et al., 2013; Bettaieb et al., 2015; Harris et al., 2015). Therefore, we evaluated whether ER stressmediated pathways are active in periodontal disease and if sEH deletion or pharmacological inhibition attenuate ER stress response. We observed the activation of the two major branches of the ER stress signaling cascade in the gingival tissue samples. While the levels of phosphorylated pPERK and pIRE1α were below the detection limit in untreated wild type and sEH

knockout mice, they were significantly elevated in the gum tissue of mice infected with *A. actinomycetemcomitans*

(Figure 5). Consistent with their phosphorylation, downstream targets for each ER stress sensor (eIF2α and spliced XBP1) were significantly elevated in infected mice. Phosphorylated eIF2α and spliced X-box binding protein 1 (sXbp1), were below the level of detection in healthy tissues but were significantly increased in mice with periodontal disease. Notably, sEH knockout mice infected with *A. actinomycetemcomitans* displayed significantly lower ER stress compared to wild type animals infected with *A. actinomycetemcomitans*. However, markers of ER stress were higher in knockout mice compared to TPPU treated wild type animals. This observation suggests that chemical inhibition of sEH was more efficacious in attenuating ER stress. On the other hand, there was no difference between TPPU alone or in the presence of EET–methyl esters at the concentrations tested ($p > 0.05$).

Figure 6.

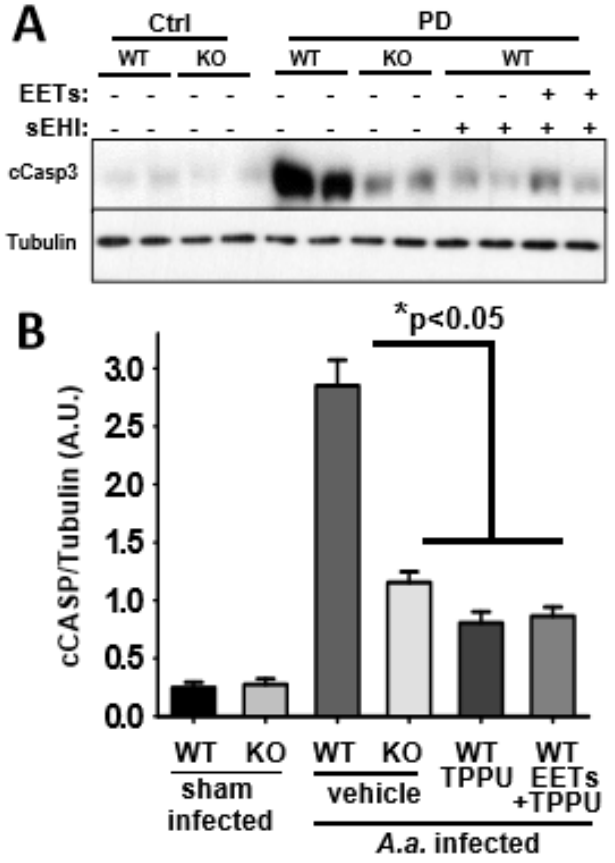

Figure 6: Genetic ablation or chemical inhibition of sEH reduces apoptosis in gingivial tissue of mice infected with *A. actinomycetemcomitans.* (A) Original blot displaying two randomly selected animals for each group. Expression level of cCaspase 3 is quantified by measuring band intensity for all six mice for each group and normalized to the expression level of α-tubulin. (B) Bar graph of mean Caspase-3 band intensity, represented as arbitrary units (mean ± SEM). (*p<0.05, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey's all pairwise multiple comparison posthoc test).

A consequence of activation of ER stress sensors is a decrease in general protein synthesis and an increase in components that assist in overcoming stress. However, if these compensatory mechanisms fail to restore/maintain homeostasis

then the cells will engage apoptosis. To determine if periodontal disease leads to intense inflammatory conditions and ER stress that compel the cells to activate apoptotic cascades we monitored the levels of cleaved Caspase-3 (Figure 6). Caspase-3 is activated by upstream caspases, and is an integrator and marker of activation of the apoptotic signaling pathway. c-Caspase-3 expression was exceedingly low under normal conditions, much like the other markers used in this study. However, infection by *A. actinomycetemcomitans* significantly increased the levels of c-Caspase-3 in all mice, but most remarkably in the vehicle control group (Figure 6). This supports the hypothesis that activated ER stress in periodontal disease is linked to cell death. In contrast, mice treated with TPPU, TPPU+EET-methyl esters or the sEH knockout mice displayed significantly lower levels of c-Caspase-3. This observation suggests that inhibition of sEH was largely able to reduce ER stress and the ensuing apoptosis. However, given the remaining 10 fold increase in c-Caspase-3 in treated groups, other inflammation associated apoptotic signaling cascades may not have been targeted by inhibition of sEH.

Discussion

The pathogenesis of periodontal disease is recognized as infection-induced inflammatory tissue destruction. At the site of tissue destruction, cytokines, and inflammatory mediators are elevated. The prevalence of periodontitis in the US adult population is estimated at over 47%. In adults aged 65 and older, 64% had either moderate or severe stages of periodontal disease (Eke et al.,
2012). This prevalence rate highlights the uniqueness of periodontal disease among other conditions. Therefore, efforts to understand the pathophysiology of the disease as well as different approaches to control it should result in tremendous health benefits. In the present study, we demonstrated that an inhibitor of sEH

largely abrogates bone loss caused by periodontal disease. This seems to be based on the effects of inhibition of sEH to decrease key osteoclastogenic molecules, as well as lowering inflammation triggered ER stress and associated apoptosis in the gingival tissue.

Epoxy fatty acid generation from ARA and other unsaturated fatty acids is the third and latest major branch of the ARA cascade. The epoxyeicosatrienoic acids seem to have mostly antiinflammatory functions that are in contrast to pro-inflammatory products of the other two branches (Capdevila et al., 1981). Multiple cytochrome P450s form epoxy fatty acids and ARA for example is converted into 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, all of which have biological activity. Early evidence on the anti-inflammatory effects of 11,12-EET by preventing tumor necrosis factor-α (TNF-α)-induced activation of NF-κB and the subsequent increase in VCAM-1 (vascular cell adhesion molecule-1) expression in mice is recently supported by other studies(Node et al., 1999; Chiamvimonvat et al., 2007). However, *in vivo,* all EET regioisomers are degraded quickly by sEH, leading to the production of metabolites known as dihydroxyeicosatrienoic acids (DHETs). Thus, in the absence of a sEH inhibitor most of the biological effects of EETs are difficult to observe. The potent inhibitor of sEH TPPU stabilizes the EETs and other epoxy fatty acids *in vivo* and promotes anti-inflammatory processes. The presumed mechanism of the therapeutic effect seems to involve homeostatic regulation of the RANK, RANKL and OPG system, a master regulator of

osteoclastogenesis. Furthermore, inhibition of sEH also decreases the chemokine MCP-1 as would be expected from the anti-inflammatory effects of sEH inhibition. Overall results are nearly identical across chemical inhibitor and global knockout

mice, highly supportive of the idea that inhibition of sEH rather than inhibitor structure specific pharmacological efficacy.

Another line of evidence reported recently also argues for the activity of EETs to prevent bone loss in a different experimental paradigm (Guan et al., 2015). In an ovariectomy induced mouse model of bone loss, administration of EETs seem to prevent bone loss through a similar mechanism in which EETs normalize the plasma RANKL to OPG ratio, while RANK levels were not reported. Instead, authors demonstrate suppression of RANK by the free acid forms of EETs, in bone marrow mononuclear cells and the RAW264.7 cell line when induced by treating the cells with RANKL. In the ovariectomized mice, the prominent mechanism of bone loss seems to be driven by decrease in OPG. In contrast, we report the levels of RANK, RANKL and OPG are all increased in gingival tissue by approximately 6-fold in response to inflammation. These differences highlight the mechanistic distinction between the ovariectomy and periodontal disease induced bone loss models. Remarkably, in TPPU treated and sEH knockout mice in our study, RANK, RANKL and OPG were normalized to nearly pre-infection levels suggesting a potential therapeutic effect. This is of course an expected difference from the earlier study given that EETs have short *in vivo* half lives and inhibition of sEH stabilizes these molecules by preventing their degradation by the sEH enzymatic activity. TPPU is easy to administer orally or in drinking water, yields high exposure and is a potent inhibitor of sEH (Rose et al., 2010). It is also important to highlight that in our study, administration of EET-methyl esters alone topically and directly onto the periodontal tissue did not prevent the inflammatory periodontal bone loss. However, the EET-methyl esters in the presence of TPPU displayed detectable changes in markers of

ER stress (Figure 5). Overall, the findings demonstrate that inhibition of sEH is a viable approach to treat complex conditions that include progressive bone loss.

An active periodontal lesion is characterized by the prominent infiltration of B and T cells (Okada et al., 1983). Adoptive transfer of $RANKL^{+}$, antigen-specific T cells induce bone loss in rat periodontal tissue that received local injection of the T-cell antigen. Furthermore, T and B cells are likely the major sources of RANKL in the inflamed gingival tissues (Kawai et al., 2006). A benefit of inhibiting the sEH in this study was the sharp decrease in tissue MCP-1 (CCL-2) level. The potent chemoattractant MCP-1 is largely seen as the driver of monocyte infiltration into the gingival tissue. Thus it is plausible that administration of TPPU or in the sEH knockout mice lower levels of MCP-1 decreased the infiltration of inflammatory cells and thereby led to the downregulation of the RANK-RANKL-OPG axis. However, it seems more likely that EETs also had a direct effect on the RANKRANKL-OPG axis because while markers of ER stress were mostly normalized, expression of RANK, RANKL and OPG were fully restored to pre-infection levels. This observation suggests that decreased inflammatory cell infiltration is a distinct effect of sEH inhibition and is an additional benefit.

Deletion of sEH and its pharmacological inhibition lead to attenuation of the ER stress response in several experimental models (Bettaieb et al., 2013; Harris et al., 2015; Inceoglu et al., 2015). This homeostatic mechanism is involved in numerous pathological conditions including viral and bacterial infection and neurodegenerative diseases (Cao et al., 2016). The ER stress system is typically activated in response to stressors including excessive amounts of unfolded proteins

in the endoplasmic reticulum. ER stress response in periodontal disease was recently reported (Domon et al., 2009; Kung et al., 2015; Yamada et al., 2015). These authors suggested that modulation of the ER stress system could have therapeutic effects. Consistent with their prediction, in this study we detected the activation of ER stress in the gingival tissue suggesting that, at the least, ER stress contributed to the development and progression of periodontal disease. The markers of activated ER stress were attenuated by sEH pharmacological inhibition and deletion. Activated ER stress signaling may lead to apoptosis when cells are unable to maintain homeostasis. This was reported in the gingival tissue of diabetic rats with periodontal disease (Kang et al., 2012). Here, we demonstrate the activation of apoptosis in conjunction with ER stress. However, blocking ER stress and inflammation with TPPU was sufficient to prevent apoptosis and this was consistently observed in sEH-/- mice inoculated with *A. actinomycetemcomitans.* These findings are consistent with those reported earlier for periodontal disease models. Specifically, in diabetic rats, *A. actinomycetemcomitans* induced a caspase3-dependent response and led to increased number of cells going through apoptosis in the gingival epithelial and connective tissues and increased bone loss (Kang et al., 2012).

Overall, the results reiterate the importance of the RANK/RANKL/OPG system and its crosstalk with ER stress signaling in periodontal disease. These interactions ultimately give rise to increased apoptosis in the gingival tissue and bone loss. Given the importance and the epidemiology of gingival diseases, therapeutics that selectively targets the elements of the host inflammatory responses should prove useful to improve oral health. Our findings strongly indicate that inhibition of sEH is one such therapeutic approach. Nearly identical results from

a small molecule inhibitor of sEH and the mice with genetic knockout of sEH support the idea that positively altering the bioactive lipid mediators including EETs is a viable approach to dampen destructive inflammation, apoptosis and bone loss in periodontal disease.

Authorship Contributions:

Participated in research design: Trindade-da-Silva, Bettaieb, Napimoga, Lee, Inceoglu,

Ueira-Vieira, Haj, Hammock

Conducted experiments: Trindade-da-Silva, Bettaieb, Goswami, Bruun, Lee,

Contributed new reagents or analytic tools: Bruun, Lee,

Performed data analysis: Trindade-da-Silva, Bettaieb, Napimoga, Lee, Inceoglu, Goswami,

Bruun, Ueira-Vieira, Haj, Hammock

Wrote or contributed to the writing of the manuscript: Trindade-da-Silva, Bettaieb,

Napimoga, Lee, Inceoglu, Goswami, Bruun, Ueira-Vieira, Haj, Hammock

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Footnotes

† Contributed equally to this work.

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1 Department of Entomology and Nematology and UC Davis Comprehensive Cancer Center, University of California, Davis, CA 95616, United States;

2 Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, 38400-902, Brazil;

3 Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas, 13045-755, Brazil;

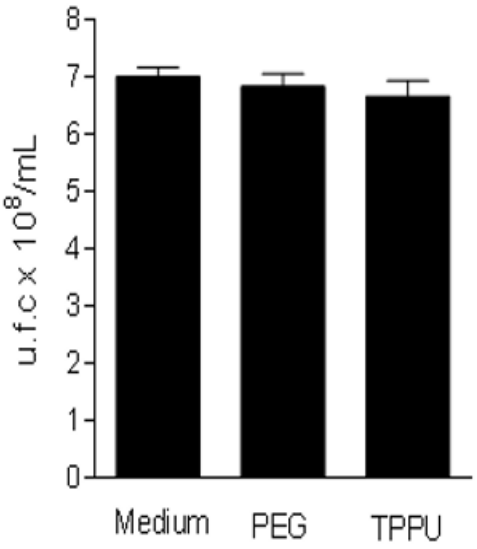
4 Department of Nutrition, University of Tennessee-Knoxville, Knoxville, TN 37996

5 Nutrition Department, University of California, Davis, CA 95616;

6 Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616, United States.

SUPPLEMENTAL FIGURES:

Supplemental Figure 1. Trindade-da-Silva, et al. **Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, RANK-related signaling, ER stress and apoptosis**_JPET



Supplemental Figure 1: TPPU or its vehicle does not affect bacterial viability. The microbial inoculum of *A. actinomycetemcomitans* was prepared and adjusted to 5 × 10^6 colony forming units (CFU)/mL in tryptic soy broth. TPPU was then dissolved to a final concentration of 10 µM in 100 % polyethylene glycol (PEG400; Fisher Scientific, Nidderau, Germany) and was added to the soy broth. Following incubation at 37°C for 24 hours under anaerobic conditions, samples serially diluted and were plated on agar plates and colony forming units were counted the next day. PEG400 solution without the inhibitor was used as a control. The results are expressed as mean ± SD.

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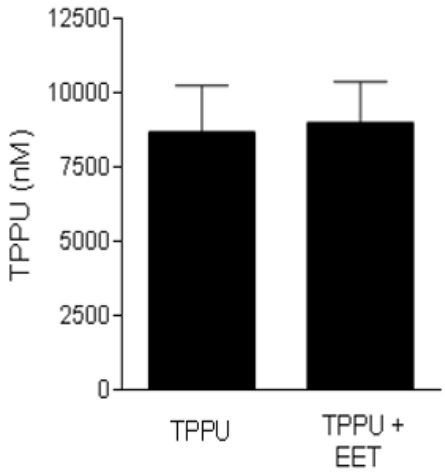
Supplemental Figure 2. Trindade-da-Silva, et al. **Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, RANK-related signaling, ER stress and apoptosis**_JPET



Supplemental Figure 2: Blood concentration of TPPU. At the end of the 15 day treatment period we quantified blood levels of TPPU from TPPU and TPPU + EET-methyl ester treated groups. The samples were taken at 24 h after the last oral gavage treatment. In both treatment groups TPPU levels were well above the theoretical IC_{90} levels by a large margin suggesting strong and systemic inhibition of sEH activity. The results are expressed as mean ± SD from six mice per group.

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Research Article

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 Induces Apoptosis and Upregulates SOCS3 in Human Thyroid Cancer Cells

Carlos Antônio Trindade-da-Silva,[1,2,3] Carolina Fernandes Reis,[1,4] Lara Vecchi,[1] Marcelo Henrique Napimoga,[5] Marcelo Sperandio,[5] Bruna França Matias Colombo,[1] Patrícia Terra Alves,[1] Laura Sterian Ward,[4] Carlos Ueira-Vieira,[2] and Luiz Ricardo Goulart[1,6]

[1]Laboratory of Nanobiotechnology, Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil
[2]Laboratory of Genetics, Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil
[3]Hammock Laboratory of Pesticide Biotechnology, University of California Davis, Davis, CA 95616, USA
[4]Laboratory of Cancer Molecular Genetics, University of Campinas, 13081-970 Campinas, SP, Brazil
[5]Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, 13045-755 Campinas, SP, Brazil
[6]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA 95616, USA

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[1]*Laboratory of Nanobiotechnology, Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil*
[2]*Laboratory of Genetics, Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil*
[3]*Hammock Laboratory of Pesticide Biotechnology, University of California Davis, Davis, CA 95616, USA*
[4]*Laboratory of Cancer Molecular Genetics, University of Campinas, 13081-970 Campinas, SP, Brazil*
[5]*Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, 13045-755 Campinas, SP, Brazil*
[6]*Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA 95616, USA*

Correspondence should be addressed to Carlos Antônio Trindade-da-Silva; carlos.biomedico@hotmail.com

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The cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a natural ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ) and a potential mediator of apoptosis in cancer cells. In the present study, we evaluated the effect of 15d-PGJ_2 in human thyroid papillary carcinoma cells (TPC-1) using different doses of 15d-PGJ_2 (0.6 to 20 μM) to determine IC_{50} (9.3 μM) via the MTT assay. The supernatant culture medium of the TPC-1 cells that was treated either with 15d-PGJ_2 or with vehicle (control) for 24 hours was assessed for IL-6 secretion via CBA assay. RT-qPCR was used to evaluate mRNA expression of IL-6, SOCS1, SOCS3, and STAT3. TPC-1 cells treated with 15d-PGJ_2 decreased the secretion and expression of IL-6 and STAT3, while it increased SOCS1 and SOCS3. Overall, we demonstrated that 15d-PGJ_2 downregulated IL-6 signaling pathway and led TPC-1 cells into apoptosis. In conclusion, 15d-PGJ_2 shows the potential to become a new therapeutic approach for thyroid tumors.

1. Introduction

Thyroid cancer combined with some of the commonest endocrine cancers shows as the 5th commonest neoplastic disease in humans, which are increasing in incidence more rapidly than any other type. The treatment of thyroid cancer consists mainly of surgical excision and ablation of the remaining tissue using radioactive iodine, which is only effective in nonmetastatic primary tumors. Metastatic disease and recurrence are mostly incurable and require advanced therapeutic strategies to improve survival [1].

The molecular pathogenesis of thyroid cancer and several signaling pathways involve signal transducers and activators of transcription (STATs), which are a family of transcription factors that regulate cell proliferation, differentiation, apoptosis, immune and inflammatory responses, and angiogenesis [2, 3]. Cumulative evidence has established that STAT3 plays a critical role in the development [4] and mediation of oncogenic signaling in many different cancers [5]. Phosphorylation of STAT3 can be induced via the stimulation of the heterodimeric receptor complex by the IL-6 cytokine family, including IL-6, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, IL-11, and cardiotrophin-1 [6]. Moreover, STAT3 phosphorylation must be precisely controlled to maintain cellular homeostasis during both

embryonic and adult development, requiring the participation of several negative regulators [7].

These negative regulators include cytoplasmic tyrosine phosphatases, for example, protein tyrosine phosphatase 1B STAT, suppressor of cytokine signaling (SOCS) proteins, which block the cytokine receptor [8]. Loss of SOCS is known to contribute to abnormal activation of STAT3 in leukemia, lymphoma, hepatocellular carcinoma, and non-small-cell carcinoma of the lung [9].

Cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), which is an endogenous molecule generated from the dehydration of PGD_2, is a natural ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ) and a potential mediator of apoptosis [10]. 15d-PGJ_2 has recently been demonstrated to exert both anti-inflammatory and antineoplastic effects in different cell lines and mouse models [11–15], although such effects have been shown to be largely independent from PPAR-γ [10], many of which are mediated via redox-modulating transcription factors, such as nuclear factor-kappaB (NF-κB), signal transducers and activators of transcription 3 (STAT3), nuclear factor-erythroid 2p45 (NF-E2) related factor (Nrf2), activator protein-1 (AP-1), hypoxia inducible factor, p53, and peroxiredoxins [16]. The electrophilic carbonyl group present in 15d-PGJ_2 cyclopentenone ring has been suggested as the main culprit for most such non-prostaglandin-like effects, since it promptly reacts with cysteine thiol groups of proteins that can be critical in the proliferative machinery of the cell [10].

Considering the cumulative evidence pointing towards a potent antineoplastic effect of 15d-PGJ_2 as well as the scarcity of studies investigating its effects on thyroid malignancies [17], the aim of this study was to evaluate the chemotherapeutic effect of 15d-PGJ_2 in thyroid cancer cells *in vitro*.

2. Materials and Methods

2.1. Cell Line. A papillary thyroid cancer (TPC-1) cell line was selected and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in humidified 5% CO_2 atmosphere at 37°C. A normal fibroblast cell line (FG11) was cultured under the same conditions and used as control for cytotoxicity.

2.2. Analysis of Cell Viability. The effect of 15d-PGJ_2 on TPC-1 viability was evaluated using the MTT assay. Briefly, thyroid cancer cells were seeded in triplicate in 96-well plates containing 200 μL of DMEM + 10% FBS (1×10^4 cells per well) and incubated with 15d-PGJ_2 at concentrations ranging from 0.6 to 20 μM for 72 hours. Cells from each well were treated with 10 μL solution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) and plates were incubated for additional 4 h at 37°C. Sulfuric acid at 2 N (200 μL/well) was added and mixed thoroughly to dissolve the dark-blue crystals. Absorbance of the converted dye was measured by spectrophotometry using a microplate reader at 570 nm (test) and 650 nm (reference). Cell survival was calculated as the percentage of MTT inhibition as follows: % survival = (mean experimental absorbance/mean control absorbance) × 100.

FG11 cells were also seeded as described above for TPC-1 cells. They were then incubated with 15d-PGJ_2 at concentrations ranging from 5 to 15 μM for 24, 48, and 72 hours. Cell count and viability were assessed on Vi-Cell XR equipment (Beckman Coulter, USA).

2.3. Evaluation of Apoptosis via Annexin V Staining. Drug-induced apoptosis was measured using Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and PI costaining using Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). After 24 hours of treatment with 15d-PGJ_2, cells were rinsed and resuspended in 100 μL of staining solution (Annexin V-FITC and PI in HEPES buffer). Cells were then incubated at room temperature in the dark for 15 min, followed by the addition of 400 μL of binding buffer. The percentage of apoptotic cells was established by flow cytometry using a FACS Accuri C6 Flow (BD eBiosciences).

2.4. Cytokine Analysis. The effect of 15d-PGJ_2 on cytokines production by TPC-1 cells was evaluated in IMDM medium from 0 to 24 hours at 37°C and 5% CO_2. This experiment was performed in triplicate using 24-well plates (1×10^4 cells/well). Cells suspensions were supplemented with 15d-PGJ_2 at 9.3 μM per well. Cytokines present in the culture supernatants were analyzed by BD Cytometric Bead Array (CBA) for Human Th1/Th2/Th17. This method uses beads with different fluorescence intensities in conjunction with a cytokine-specific capture antibody. Measurements were performed using FL2 and FL3 channels of the Flow Cytometer Accuri C6 Flow (BD eBiosciences). A specific detection kit for IL-6 was used according to the manufacturer's protocols (BD eBioscience). Analysis output was obtained in the form of tables and charts using the FCAP Array™ Version 3.0 Software (BD eBioscience).

2.5. mRNA Expression Analyses. Quantitative real-time PCR (RT-qPCR) assays were performed using the Applied Biosystems 7500 Sequence Detecting system (Applied Biosystems, California, USA) and SYBR Premix Ex Taq II (Takara, Shiga, Japan) under the following reaction conditions: 40 cycles of PCR (95°C for 15 s and 60°C for 1 min) after an initial denaturation (95°C for 1 min). The primers used for amplification were as follows: SOCS3, Forward: TCACCGAAAACACAGGTTCCA and Reverse: GAGTATGTGGCTTTCCTATGCTGG; β-actin, Forward: CTACAATGAGCTGCGTGTGGC and Reverse: CAGGTCCAGACGCAGGATGGC. Amplification of the housekeeping gene β-actin was used as an internal control to normalize the SOCS3 mRNA level. The RT-qPCR data were presented as cycle threshold levels and were normalized against the corresponding β-actin control cycle threshold values. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, as described previously [18].

2.6. Statistical Analysis. The data were analyzed on GraphPad Prism (v.6.0c) software to compare the effects of different

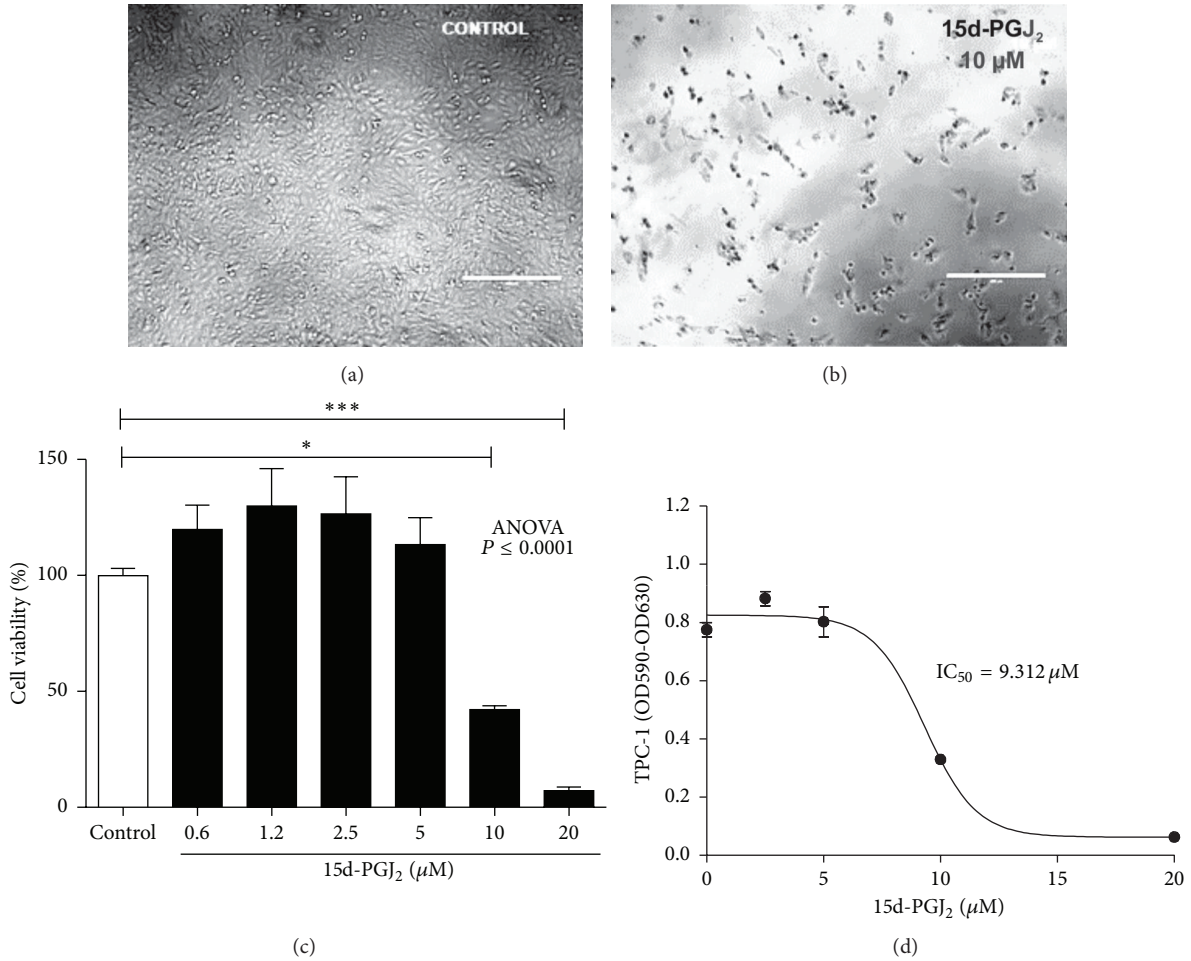

Figure 1: 15d-PGJ_2 decreased the viability of TPC-1 cells. TPC-1 cells were treated with 15d-PGJ_2. (a) represents the cell culture without treatment. (b) Cells treated with 10 μM of 15d-PGJ_2. (c) Viability of the TPC-1 cells treated with 15d-PGJ_2 in the concentrations of 0 to 20 μM. (d) IC_{50} from cell viability following treatment with 15d-PGJ_2. The data are presented as means ± standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control ($^{*}P > 0.01$; $^{***}P > 0.001$).

treatments. Two-way ANOVA and Bonferroni's *post hoc* tests were used to analyze the data.

3. Results

3.1. In Vitro Effect of 15d-PGJ_2 on TPC-1 and FG11 Cell Proliferation and Viability. 15d-PGJ_2 decreased cell proliferation (Figures 1(a) and 1(b)) and cell viability at the concentrations of 10 μM and 20 μM (Figure 1(c)). These findings were used to calculate IC50, which was established at 9.3 μM (Figure 1(d)). This concentration was then used for subsequent experiments. 15d-PGJ_2 did not show significant effect on fibroblast proliferation and viability in doses varying from 5 to 15 μM (Figure 2).

3.2. Apoptotic Effects of 15d-PGJ_2 on Thyroid Cancer Cells. The Annexin V apoptosis assay on TPC-1 showed that 47% of the cells treated with 15d-PGJ_2 (9.3 μM) entered apoptosis, whereas less than 5% were observed in the control group (Figure 3).

3.3. Relative IL-6 mRNA Expression and IL-6 Release by TPC-1. The results revealed that IL-6 was highly expressed in TPC-1 and treatment with 15d-PGJ_2 decreased the relative IL-6 mRNA expression after 4 hours (Figure 4(a)). Concurrently, IL-6 release in the cell culture medium increased at a much lower rate than in the control group, thus demonstrating the downmodulation effect of 15d-PGJ_2 on IL-6 secretion by TPC-1 cells as soon as two hours after treatment (Figure 4(b)).

3.4. Relative Expression of SOCS3, SOCS1, and STAT3. Upregulation of SOCS1 and SOCS3 occurred rather early in TPC-1 treated with 15d-PGJ_2 (Figures 5(a) and 5(b)). A significant difference between the control and the treated cells was observed two hours after treatment, with SOCS3 showing a fourfold increase in relative mRNA expression. Such an effect was not long-lasting, and 4 hours after treatment the expression of SOCS1 and SOCS3 was normalized. STAT3 was downregulated 4 hours after treatment and was maintained throughout the assay for 24 hours (Figure 5(c)).

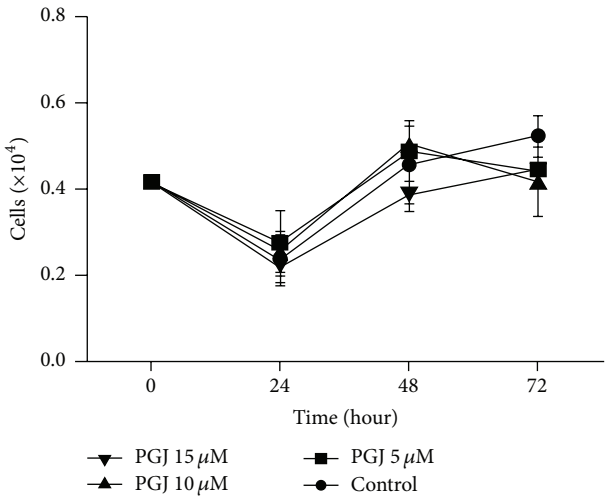

FIGURE 2: Fibroblast (FG11) cell proliferation under 15d-PGJ_2 treatment. FG11 cells were treated with 5 to 15 μM of 15d-PGJ_2. The data are presented as means ± standard deviation of three replications from at least three independent tests. 15d-PGJ_2 did not show significant difference from the control at the doses of 5 μM, 10 μM, and 15 μM.

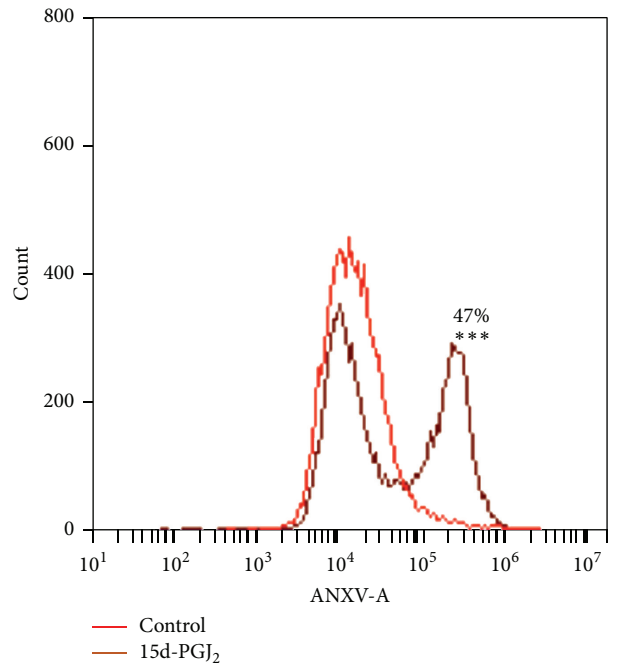

FIGURE 3: 15d-PGJ_2 induced apoptosis in TPC-1 cells. The Annexin V assay revealed that 15d-PGJ_2 induced 47% apoptosis in TPC-1 compared to 5% in the control group. The data are presented as means ± standard deviation of three replicates from at least three independent tests. ***Statistically significant difference from the control ($P > 0.001$).

4. Discussion

The most important adverse aspects in the current surgical approach to treat papillary thyroid carcinoma is the risk of long-term recurrence and the difficulty in managing metastatic disease, especially in those cases initially regarded as low risk [19, 20]. In the recent past, great efforts have been made to define new molecular therapies to potentiate the effectiveness of current cytostatic drugs and 15d-PGJ_2 has recently emerged as a potent antineoplastic molecule [21].

Several studies have demonstrated that although 15d-PGJ_2 is an endogenous ligand of PPAR-γ, most of its antineoplastic effects are PPAR-γ-independent [22, 23]. The effects of PPAR-γ ligands may also act by independent mechanisms because they differ widely amongst carcinoma types and thus must be individually examined.

The present study investigated the role of exogenous 15d-PGJ_2 on papillary thyroid carcinoma cells, the TPC-1 cell line. The drug reduced cell viability at the doses of 10 and 20 μM (Figure 1(c)). Similar results have been found in cell viability in cultures with other cell lines of breast cancer, lung cancer, lymphoma [24, 25], and colorectal [26, 27], ovarian [22], gastric [21], pancreatic [28], and prostate cancer [29].

Despite the overall antitumoral effect of 15d-PGJ_2, most studies have reported both dose and time-dependent responses, with lower doses often promoting opposing effects to the cytotoxic doses [23]. Micromolar doses of 15d-PGJ_2 are required to induce lymphoma cell death [30, 31], whereas physiological concentrations of the metabolite are in the range of picomolar to nanomolar [23, 32]. It has also been reported that high doses of 15d-PGJ_2 (≥ 5 μmol/L) caused cytotoxicity in cultured neurons, whereas low concentrations of the agonists (15d-PGJ_2, ≤ 1 μmol/L) suppress rat and human neuronal apoptosis and necrosis induced by H_2O_2 treatment [32].

Production of IL-6 and signaling are prerequisites for tumor progression [33]. Indeed, the overproduction of IL-6 is commonly encountered in a variety of cancer cells and elevated serum IL-6 levels correlate with poor outcome in cancer patients [34–36]. IL-6 was shown to be an autocrine proliferation factor for tumor cell lines [37–39]. Additionally, STAT3 has been reported to be overexpressed in nearly 40% of all breast carcinomas due, in part, to autocrine expression of IL-6 [40]. In turn, paracrine IL-6 can induce autocrine IL-6 expression in cells within the tumor microenvironment, thus establishing an IL-6^+ niche and enhancing tumor progression [35]. The TPC-1 cells treated with 15d-PGJ_2 in the current study have shown a decrease in IL-6 expression and release associated with reduced cell proliferation, thus corroborating the aforementioned mechanism of IL-6-linked neoplastic progression in thyroid cancer cells. Recent studies have corroborated the inhibitory effect of 15d-PGJ_2 on IL-6 expression both *in vitro* [41] and *in vivo* [42].

Being different from normal cells, which phosphorylate STAT under stringent control, STAT3 is continuously phosphorylated in several neoplastic diseases via the overproduction of agonists, such as specific cytokines, namely, IL-6, and their respective cytokine receptors [40]. This cycle can be further enhanced via antagonism of negative regulators, such as SOCS and tyrosine phosphatases [43].

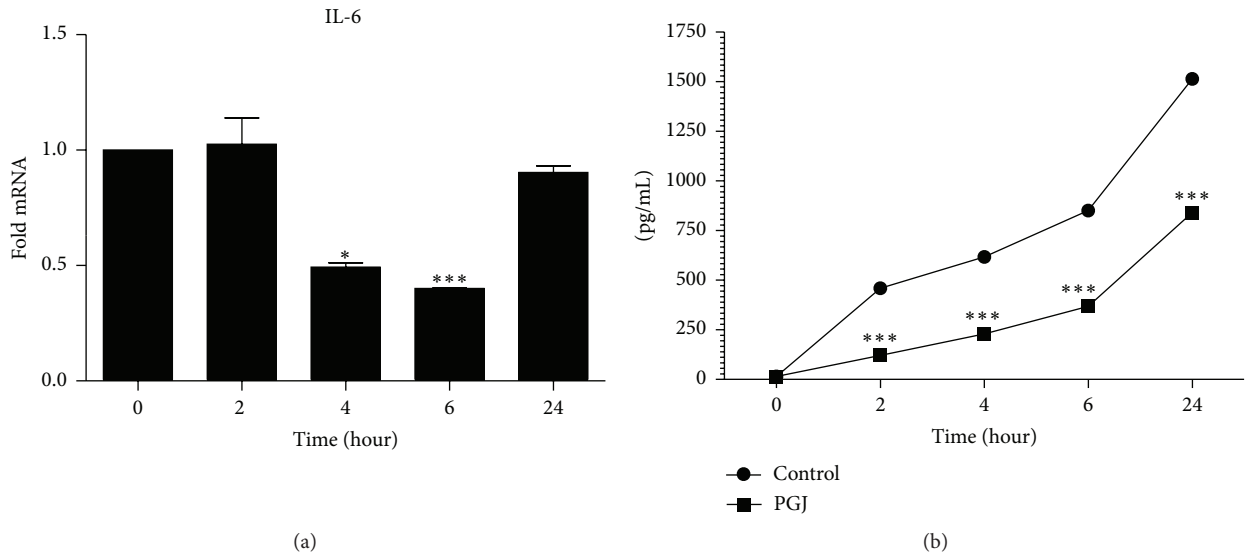

Figure 4: Decreased relative IL-6 mRNA expression and release, TPC-1 cells treated with 15d-PGJ_2. TPC-1 cells were treated with 15d-PGJ_2 (9,8 μM) for 0 to 24 h. (a) shows the relative IL-6 expression. (b) Quantitative IL-6 released by TPC-1 cells treated with 15d-PGJ_2 against the control group. The data are presented as means ± standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control group ($^{*}P > 0.01$; $^{***}P > 0.001$).

STAT3 has been reported to play an important role in maintaining cancer stem cells both *in vitro* and *in vivo*, implicating an integral involvement of STAT3 in tumor initiation, progression, and maintenance [4]. In fact, this signaling route is so relevant in tumorigenesis where targeting STAT3 in neoplastic bone marrow disease practically interrupted the progression of metastasis [44–47]. Cumulative evidence points to a clear STAT3-inhibitory effect of 15d-PGJ_2 in inflammatory diseases [10, 48, 49]. However, our findings show a small and stable decrease in the relative expression of STAT3 in thyroid cancer cells treated with 15d-PGJ_2 (Figure 5(c)), although not significant. It is possible that STAT3 phosphorylation was prevented by 15d-PGJ_2 through the upregulation of SOCS3, which results in the inhibition of STAT3 activation, as shown elsewhere [50].

Upregulation of both SOCS3 and SOCS1 was also followed by the downregulation of IL-6 expression in TPC-1 cells related to the exposure to 15d-PGJ_2. SOCS3 is an inducible endogenous negative regulator of STAT3, and it is suggested as a tumor suppressor gene [51]. Negative modulation of SOCS1 and SOCS3 is a survival strategy in most cancer cells [52–54]. Conversely, overexpression of such cytokine inhibitors may indicate an antiproliferative response. Indeed, our results have demonstrated that 15d-PGJ_2 increased SOCS3 on TPC-1 cells within two hours of contact with the drug, thus supporting the antioncogenic nature of this gene (Figure 5(b)). Interestingly, cells presented diminished levels of SOCS3 and SOCS1 six hours after treatment, which was extended to 24 hours after treatment (Figures 5(a) and 5(b)), probably because 15d-PGJ_2 was already driving cells into apoptosis (Figure 3).

Regarding the downregulation of IL-6 mediated by SOCS3 overexpression, as early as two hours after exposure to 15d-PGJ_2, and considering the detrimental effects and actions of IL-6 linked with tumor growth, progression, and relapse [55–57], 15d-PGJ_2 is presented as a novel antineoplastic drug.

Our data demonstrated that apoptosis was detectable in nearly 50% of the TPC-1 cells treated with 15d-PGJ_2, compared to 5% in the control group. We have also demonstrated that SOCS3 overexpression was an early event in treated cells, while STAT3 remained stable over 24 hours. It is known that the activation of STAT3 in cancers leads to gene expression promoting cell proliferation and resistance to apoptosis [58], but 15d-PGJ_2-induced SOCS3 overexpression may have prevented STAT3 phosphorylation [50]. Despite the premature and short-lasting effect of 15d-PGJ_2 on SOCS3, its expressive upregulation (Figure 5(a)) may have been high enough to mediate apoptotic signaling within cells [59].

5. Conclusion

The present study shows important antiproliferative and apoptotic activities in human thyroid cancer cells induced by 15d-PGJ_2. Such events are linked with the overexpression of SOCS3 that inhibits IL-6 signaling, a key factor in many cancers. This is the first report on 15d-PGJ_2-induced SOCS3 expression, which evidences a novel therapeutic option for the treatment of thyroid cancer and other cancers that are dependent on IL-6 signaling.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

Carlos Antônio Trindade-da-Silva and Carolina Fernandes Reis have equally contributed to this work.

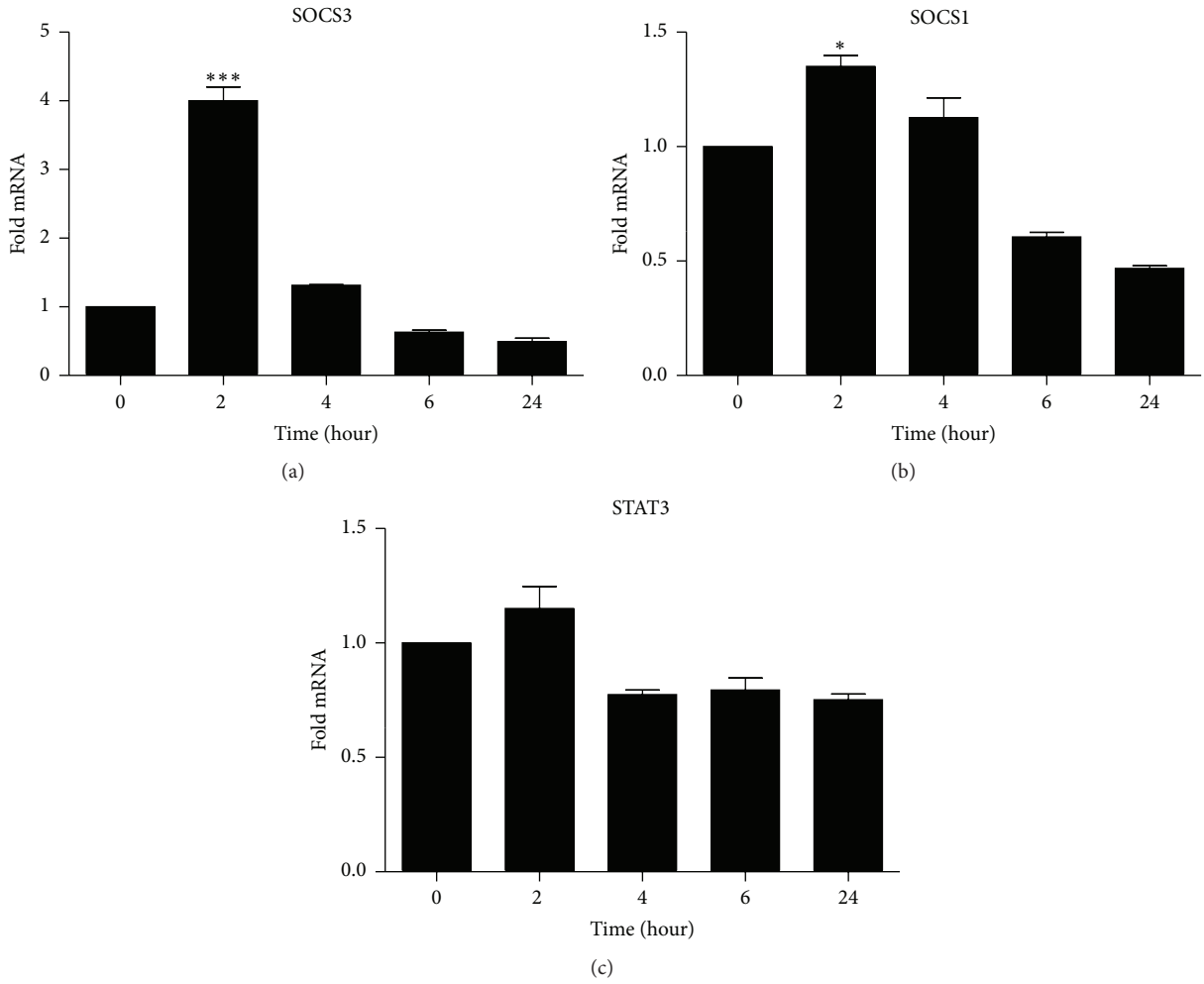

Figure 5: SOCS3 and SOCS1 increased in TPC-1 cells treated with 15d-PGJ_2. TPC-1 cells were treated with 15d-PGJ_2 (9,8 μM) for 0 to 24 h. (a) shows the relative expression of SOCS3 (b), SOCS1 (c), and STAT3 (c) in the first two hours of treatment and decreased STAT3 four hours after the treatment (c). The date are presented as means ± standard deviation of three replicates from at least three independent tests. An asterisk * indicates statistically significant difference from the control ($^{*}P > 0.01$; $^{***}P > 0.001$).

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