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Oxidative actions of hydrogen peroxide in human gingival and oral periosteal fibroblasts: Responses to glutathione and nicotine, relevant to healing in a redox environment☆

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ABSTRACT

Background: This study aims to validate pro-oxidant actions of nicotine (N), using hydrogen peroxide (H_2O_2) and the antioxidant glutathione (G) in an *in vitro* model of human gingival fibroblasts (HGF) and human oral periosteal fibroblasts (HPF); radiolabelled androgens are used as biomarkers of redox status. Oxidative stress is an important mediator of inflammatory repair. The androgen metabolite 5α-dihydrotestosterone (DHT) is an effective biomarker of oxidative stress and healing.

Methods: 6 Cell-lines of HGF and HPF established in confluent monolayer culture were incubated in Eagle's MEM using 14C-testosterone and 14C-4-androstendione as substrate; in conjunction with effective concentrations of N, G and H_2O_2 established at N250, G3 μg/ml and 3%H_2O_2 w/w, 0.5 μl/ml. Combinations of H_2O_2G and H_2O_2GN were used in order to compare the oxidative effects of N/H_2O_2 and their responses to glutathione. At 24 h, the medium was solvent extracted, evaporated to dryness and subjected to TLC in a benzene/acetone solvent system 4:1 v/v for the separation of metabolites. The separated metabolites were quantified using a radioisotope scanner.

Results: The mean trends of 6 cell-lines for both substrates and each cell type demonstrated that the yield of the main metabolite DHT was significantly reduced by N and H_2O_2 alone (2-fold, $n=6$; $p<0.01$). The inhibition caused by H_2O_2 was overcome by the antioxidant glutathione in the combination H_2O_2G, to values similar to those of controls ($n=6$; $p<0.01$). It is relevant that when N was added to this neutralized combination, the decrease in yields of DHT triggered by N were comparable to those induced by H_2O_2; and retaining the positive effect of G.

Conclusion: Oxidative stress mediated by H_2O_2 was overcome by glutathione and recurred when nicotine was added, suggestive of a pro- oxidant role for nicotine. Androgen biomarkers are a sensitive index of oxidative stress which affects wound healing.

Introduction

The aim of this investigation is to study the modulatory effects of the antioxidant glutathione on the oxidative effects of hydrogen peroxide (H_2O_2), in order to validate pro-oxidant actions of nicotine in human gingival fibroblasts (HGF) and human oral periosteal fibroblasts (HPF). Steroid hormones are effective biomarkers of matrix synthesis and oxidative stress. These actions have applications in our investigation in utilising radiolabelled androgen substrates 14C-testosterone and 14C-4-androstenedione for *in vitro* assays of metabolic markers formed in response to agents tested in cell culture; in the context of wound healing in a redox environment, using hydrogen peroxide (H_2O_2), nicotine (N) and glutathione. The rationale for using the agents tested is addressed below, in order to coordinate with wound healing and antioxidant actions of androgen metabolites used as markers in our cell culture experiments.

Androgen biomarkers

The biologically active androgen DHT has antioxidant properties and it is an effective marker of oxidative stress [1]. It is significant that androgen hormones trigger antioxidant enzyme action via relevant gene activation [2], demonstrating applications as redox markers. They also have direct antioxidant actions [3]. DHT induces anti-apoptotic proteins and reduces oxidative stress in a redox environment [4]. Androgen receptor (AR) proteins directly activated by DHT play an important role as redox regulators via direct actions on glutathione S-transferase [5].

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Physiological concentrations of T and DHT have been shown to increase endothelial synthesis of NO due to rapid recruitment of extracellular signal-related kinase and the phosphotidylinositol 3-OH kinase/Akt cascades resulting in phosphorylation of endothelial nitric oxide synthase [6]. These actions of DHT are exerted via AR; and explain the impact of androgens in an oxidative stress-inducing environment. Novel androgen-responsive genes identified in gene expression libraries are associated with protein synthesis, oxidative stress responses, transcription, proliferation, apoptosis and differentiation [7]. These molecular mechanisms demonstrate the relevance of androgen responsive genes and androgen metabolites as biomarkers of oxidative stress and wound healing, utilized in our experiments.

Receptors for androgens have been detected in fibroblasts derived from periodontal and gingival connective tissue [8] suggestive of androgen mediated activity in gingivae and the periodontium. Specific inhibition of androgen 5α-reductase by the inhibitor finasteride in human gingival [9] and oral periosteal [10] fibroblasts is suggestive of androgen target tissue activity in these cells. Physiologically active androgen metabolites are useful biomarkers of oxidative stress- related anabolic activity applicable to healing; used in this context in our experiments.

Rationale for using nicotine, glutathione and hydrogen peroxide

There is documented evidence of the detrimental effects of nicotine on wound healing. Delivery of nicotine in a rat model caused significant down-regulation in the expression levels of osteopontin, type ll collagen, bone morphogenic protein-2, bone sialoprotein and core-binding factor α-1; compared with controls [11]. These findings are suggestive of inhibition of bone matrix-related gene expression required for bone healing. Cytotoxic effects of nicotine/tar-free cigarette smoke in glioma cells are overcome by *N*-acetyl-L-cysteine and reduced glutathione [12]. Membrane damage is prevented by catalase and endaravone, an OH scavenger. In contrast, cell apoptosis does not respond to endaravone; and is induced by the authentic hydrogen peroxide (H_2O_2) system generated by the xanthine/xanthine oxidase system, indicating the importance of hydrogen peroxide in cell apoptosis. These finding imply different oxidative systems associated with membrane damage and apoptosis, worthy of investigation in a cell culture model.

Changes in cell viability and increased generation of ROS in response to nicotine have been demonstrated in human gingival fibroblasts [13], of relevance to periodontitis. There is morphological evidence of increased apoptosis with transient activation of JNK, ERK and concomitant activation of P38; evidenced by CASP3 activation and cleavage of poly ADP ribose polymerase (PARP). These findings indicate that nicotine-induced apoptosis in HGF occurs via generation of ROS and CASP3-dependent pathways. Investigation of the actions of nicotine in response to androgen receptor-mediated pathways would provide a further dimension to the mechanisms involved.

Nicotine-injected rats demonstrate significant elevation in levels of malondialdehyde (MDA), a marker of oxidative stress; it is a naturally occurring product of lipid peroxidation and prostaglandin synthesis [14]. Nicotine induced a significant negative correlation between reduced glutathione and MDA. These findings demonstrate oxidative effects of nicotine, overcome by supplementation of the diet with epicatechin and vitamin E. In our study, glutathione serves as an antioxidant to be tested for androgen responsiveness in the *in vitro* cell culture model used.

Redox agents, human gingival and oral periosteal fibroblasts in culture

Cells in culture are sensitive to oxidative stress-inducers. Ascorbate and phenolic compounds are oxidized to generate H_2O_2 in cell culture. Decreased levels of H_2O_2 have been detected when oxaloacetate is added, resulting in its depletion. These observations raise important issues regarding the behaviour and metabolism of cells in culture which are sensitive to oxidative stress in this environment [15]. H_2O_2 affects barrier function by changing the location of claudin-4 protein from an insoluble to a soluble fraction and from an apical tight junction to a lateral membrane, in colonic epithelium [16]. The modulation of androgen metabolism by nicotine and H_2O_2 and responses to glutathione as an index of healing is a pertinent area of investigation due to the potential role of oxidants and antioxidants in mediating the actions of nicotine. The metabolic activity of testosterone is greater in subcellular fractions of inflamed gingivae than in healthy fractions [17] suggestive of a reparatory role for androgens in fibroblasts from an inflamed source.

Cultured autogenous periosteal cells have applications for alveolar bone regeneration. When mixed with platelet-rich plasma and particulate autogenous bone prior to grafting, satisfactory bone regeneration is seen despite severe atrophy of the alveolar process. There is significant recruitment of osteoblasts and osteoclasts associated with angiogenesis around regenerated bone with a more rapid remodelling process than that associated with conventional bone grafting [18], suggestive of the periosteum being a good source of osteoprogenitor cells. Bone remodelling induced by cultured autogenous periosteal cells, have several applications for enhanced osseointegration.

In our investigation we have used fibroblasts from chronically inflamed gingivae and periosteum. Human gingival and oral periosteal fibroblasts are used in culture to compare the oxidative actions of H_2O_2 with nicotine in response to the antioxidant glutathione using androgen biomarkers of redox healing; using a novel metabolically active cell culture model, enabling closer extrapolation to *in vivo* conditions.

Materials and methods

Radiolabelled androgens (50 μCi each of 14C-testosterone and 14C-4-androstenedione of specific activity 58 μCi/μM) were obtained from Amersham International, Amersham, Bucks, UK. Ethyl acetate for solvent extraction of metabolites, benzene, acetone and chloroform used for thin layer chromatography (TLC) as well as pre-coated silicagel kiesegel 60 TLC plates were supplied by BDH Chemicals (Merck), Dagenham, Essex. Cell culture materials including Eagle's MEM, FBS, L-glutamine, antibiotic solution (penicillin/streptomycin), sodium bicarbonate and cell culture plastics were purchased from Invitrogen Ltd. Glutathione, H_2O_2 and nicotine used in the incubations; and 0.4% Trypan Blue solution (w/v) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Methods

Human gingival and oral periosteal fibroblasts were derived from chronically inflamed gingivae and oral periosteal tissue of patients aged 30–50y attending Periodontology, King's College London Dental Hospital, UK, for periodontal surgical procedures. Ethical permission was obtained from King's College Hospital Ethics Committee. In addition, written informed consent was obtained from each patient for the surgical procedure; this included written informed consent from the donor for the anonymous use of tissue samples in research, as required by the Hospital Trust. Chronically inflamed gingival tissue was isolated during pocket elimination surgery of 6–8 mm sites, after completion of initial phase periodontal treatment; this tissue is usually discarded. Oral periosteal tissue was isolated during mucogingival procedures from the bone surface, beyond the mucogingival junction.

It is known that fibroblasts from chronically inflamed tissue metabolise androgen substrates better than those from a non-inflamed source [19]. Other studies have shown that chronically inflamed tissue from both genders show no significant difference in metabolizing testosterone, when compared with non-inflamed tissue; where testosterone is metabolized to a greater extent by tissue obtained from males [17]. Based on these findings, the study samples were not characterized for the sexes, although we used both radiolabelled testosterone and 4-androstenedione as substrates, the latter being the predominant circulatory hormone in females; it is converted to testosterone via 17β-hydroxysteroid dehydrogenase activity and subsequently to DHT as a result of 5α-reduction. In addition, test and control cell sources were age and sex matched.

The samples were not pooled, maintaining individual cultures for cell-lines derived from 4 to 6 subjects of 40–50y, each with its own control, matched for age and gender; values represent means from cell-lines derived from 4 subjects, (experiments for establishing optimal concentrations of hydrogen peroxide) or 6 subjects (experiments using optimal concentrations of H_2O_2, glutathione or nicotine, alone and in combination) studied under the same conditions. Replicate incubations were performed with cell-lines derived from 4 to 6 subjects and results are presented as mean values of all cell-lines used ($n=4$; $n=6$); with standard deviations from the mean.

Primary explants of gingival/periosteal fibroblasts were established by incubation of 1 mm^3 minced gingival tissue/oral periosteal tissue with Eagle's MEM. Serial passaging of primary explants was carried out for each of the 6 cell-lines established, by trypsinising confluent cells with 0.25% trypsin. Cells of the 5th–9th passage were used for experiments with the 6 cell-lines thus established.

For each cell-line, fully confluent fibroblasts in 25 cm^2 flasks (2.2×10^6 cells) were divided amongst 24 wells of a multiwell plate and experiment were set up to establish the optimal concentrations of H_2O_2 ($n=4$) and to study the effects of optimal concentrations of H_2O_2, glutathione, previously established at 3 μg/ml (G) and nicotine, previously established at 250 μg/ml (N), alone and in combination ($n=6$). Human gingival fibroblasts (HGF) and human oral periosteal fibroblasts (HPF) were used with androgen substrates 14C-testosterone and 14C-4-androstenedione as described below.

1. *Establishing optimal effective concentrations of H_2O_2*
   The stock solution consisted of 100 μl of stabilized H_2O_2 30% (w/w). This solution was diluted 10-fold (in 1000 μl of medium) in order to dispense small concentrations of hydrogen peroxide in each well. Serial concentrations of 0.1, 0.5, 1, 2 and 3 μl of 3% (w/w) H_2O_2 were dispensed per well, using 4 cell lines of HGF and 4 cell-lines of HPF for each of the substrates, 14C-testosterone and 14C-4-androstenedione.
2. *Effects of optimal concentrations of glutathione (G), hydrogen peroxide (H_2O_2), nicotine (N) and their combinations (H_2O_2G, H_2O_2GN) on the metabolism of two androgen substrates by HGF and HPF*
   The effective concentration of H_2O_2 was established as indicated above, at 0.5 μl/ml of 3% H_2O_2 (w/w). Each of 6 cell-lines of HGF and HPF were incubated in Eagle's MEM, with the substrates 14C-testosterne/14C-4-androstenedione, in the presence or absence of effective concentrations of 3% H_2O_2 w/w at 0.5 μl/ml, nicotine (N250 μg/ml) and glutathione (G3 μg/ml) as established previously; including combinations of H_2O_2G and H_2O_2GN, in order to compare the oxidative effects of N/H_2O_2 and their responses to glutathione.
   For all experiments, at the end of a 24 h incubation period, the medium was solvent extracted with ethyl acetate (2 ml × 2). The eluent containing radiolabelled androgen metabolites was subsequently evaporated to dryness in a vortex evaporator and solubilized in chloroform prior to separation by TLC in a benzene: acetone solvent system (4:1 v/v). The separated metabolites were tentatively identified using mobilities of cold standards, disclosed in iodine, confirmed by autoradiography; and quantified using a radioisotope scanner. The identity of metabolites was confirmed by gas chromatography–mass spectrometry (g.c–m.s) as described below.
   In order to ascertain cell viability at the end of the incubation period, the Trypan Blue dye exclusion procedure was carried out. Following removal of the incubation medium, a 0.4% solution of Trypan Blue (w/v), was pipetted gently to cover the basal cell layer of the multiwall plates and examined under the microscope. The lack of uptake of dye by the cells; leaving them colourless, confirmed cell viability.

Characterization of steroid metabolites by g.c–m.s

As DHT is the most significant biologically active metabolite in stimulating fibroblast matrix synthetic activity, it was characterized as follows. Several incubations were performed with human gingival fibroblasts and unlabelled testosterone (10^{-6} M). After extraction, the identity of 5α-DHT as a metabolite in the dried extracts was confirmed by combined capillary gas chromatography–mass spectrometry (g.c–m.s; courtesy of Professor A.I. Mallet, St. Thomas' Hospital, London, UK). The derivatised biological material as the pentafluorobenzyloxime trimethylsilylether (PFBO/TMS) had a molecular ion (5 5 7) and mass spectral fragmentation pattern identical to those of authentic PFBO/TMS ether of 5α-DHT, but at lower levels due to smaller concentrations of the steroid. Characteristic ions were noted, for example at m/z values of 542 [M-15]+ due to loss of a methyl group; 467 [M-90]+ due to loss of TMS ether; 452 [M-90-15]+ due to loss of TMS ether plus a methyl group and an m/z value of 360, due to loss of the pentafluorobenzyloxime group. All these procedures have been described in detail [20].

Statistical analysis

The mean values from replicates of each cell-line and standard deviations for metabolic yields from four or six such cell-lines are shown in the Results section. The cell-lines were not pooled. Significance testing was done using one way ANOVA. The unit of analysis was the subject, where the control incubation in the absence of testing agents served as the comparison for the cell-lines studied. Since individual controls were set up for each cell-line, the data is representative of the number of cell-lines studied.

Results

The main metabolites formed in the cell culture models described above were diol, DHT, androstanedione and 4-androstenedione/testosterone, with the two substrates (14C-T; 14C-4-A) used. For the purpose of clarity the metabolic yields of the physiologically relevant marker DHT only, are addressed in the context of pro-oxidant/antioxidant capacities of testing agents.

Yields of DHT in response to serial concentrations of H_2O_2 in gingival (HGF) and periosteal fibroblasts (HPF), using 14C-testosterone and 14C-4-androstendione as substrates (Fig. 1).

When human gingival fibroblasts were incubated with two radiolabelled substrates, H_2O_2 caused significant reduction in the

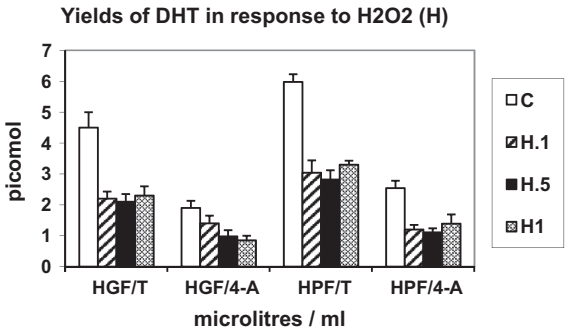

Fig. 1. Yields of DHT in response to serial concentrations of H_2O_2 (H) in HGF and HPF using 14C-Testosterone (T) and 14C-androstenedione (4-A) as substrates. Yields of DHT for each cell type and each substrate (HGF/T, HGF/4-A; HPF/T, HPF/4-A) are shown in response to serial concentrations of 3% H w/w (0.1, 0.5 and 1 ml/ml). C: Control in the absence of agents, $n=4$; $p<0.01$. Standard deviations of means are shown in all figures. Cells from a fully confluent 25sq.cm flask (2.2×10^6 cells) were divided amongst 24 wells of a multiwell plate for all experiments in Figs. 1–5; yields of DHT represent cells/well. H_2O_2 is represented as H in all figures, for clarity.

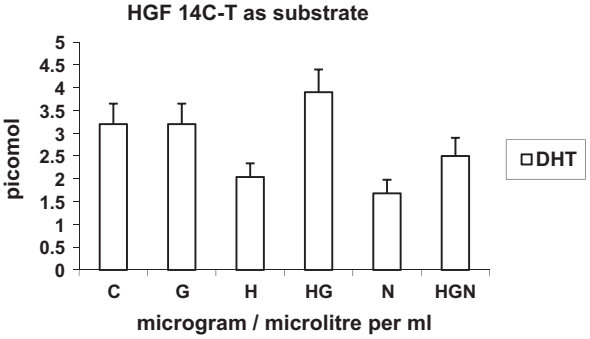

Fig. 2. Yields of DHT in response to optimal concentrations of G3, (H_2O_2) H 0.5, HG, N250 and HGN in gingival fibroblasts using 14C-testosterone as substrate. Yields of DHT are shown in human gingival fibroblasts (HGF) in response to the above agents and their combinations using 14C-T as substrate; C: Control, $n=6$; $p<0.01$.

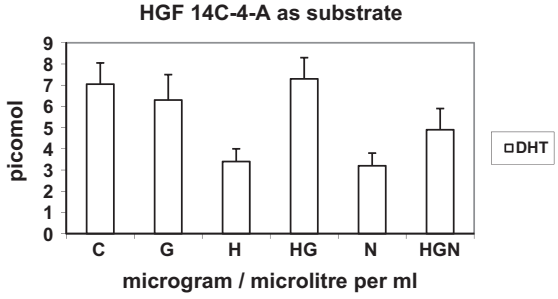

Fig. 3. Yields of DHT in response to optimal concentrations of G3, (H_2O_2) H 0.5, HG, N250 and HGN in human gingival fibroblasts (HGF) using 14C-4-androstenedione (14C-4-A) as substrate. Yields of DHT from HGF are shown as in Fig. 2 in response to the above agents tested, using 14C-4-A as substrate. C: Control, $n=6$; $p<0.01$.

yields of DHT (2-fold and 50% respectively) at a range of concentrations of H_2O_2, 0.1, 0.5 and 1 μl/ml of a 3% solution w/w (Fig. 1 $n=4$; $p<0.01$). Similarly when oral periosteal fibroblasts were incubated with two radiolabelled substrates, a 2-fold reduction in yields of DHT was maintained for both substrates at concentrations of H_2O_2, 0.1, 0.5 and 1 μl/ml, compared with control values (Fig. 1; $n=4$, $p<0.01$). In view of these results, 0.5 μl/ml of 3% H_2O_2 w/w was used as the effective concentration for subsequent experiments.

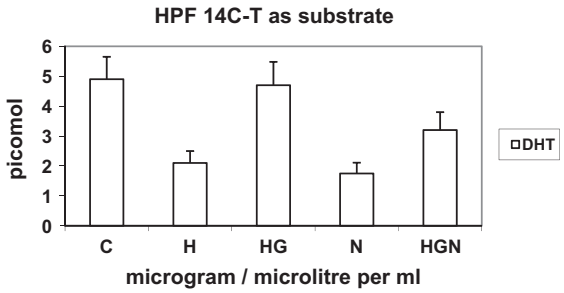

Fig. 4. Yields of DHT in response to optimal concentrations of G3, (H_2O_2) H 0.5, HG, N250 and HGN in human periosteal fibroblasts (HPF) using 14C-testosterone (14C-T) as substrate. Yields of DHT from HPF are shown in response to the above agents, using 14C-T as substrate. C: Control, $n=6$; $p<0.01$.

Yields of DHT from HGF in response to optimal concentrations of glutathione (G3), hydrogen peroxide (H_2O_2 0.5), nicotine (N250) and their oxidant antioxidant combinations H_2O_2G and H_2O_2GN; using 14C-testosterone/14C-4-androstenedione as substrate (Figs. 2 and 3).

a. When human gingival fibroblasts were incubated with 14C-testosterone and the above agents, the main metabolites formed were DHT, diol and 4-androstenedione. The results for DHT are shown (Fig. 2). Glutathione alone showed similar results to those of controls. There was a 37% reduction in the yield of DHT in response to H_2O_2, restored to values marginally above C when G was added to the incubation. N alone caused a 90% reduction in DHT. When nicotine was added to the neutralized incubation, there was a reduction of 36% in the yield of DHT, suggestive of a mechanism similar to that of H_2O_2. These results were all significant (Fig. 2; $n=6$, $p<0.01$).
b. When 14C-4-androstenedione was used as substrate the main metabolites formed were DHT, diol and testosterone. The results for DHT are shown (Fig. 3). G alone caused a marginal reduction in the yield of DHT when compared with controls. H_2O_2 caused a 2-fold reduction in yields of DHT over control incubations; while addition of the antioxidant glutathione increased the yields to those similar to controls. Nicotine alone reduced yields by 2.2-fold over C and the addition of N to the neutralized oxidant/antioxidant incubation H_2O_2G, reduced yields by 33% (Fig. 3; $n=6$, $p<0.01$).

Yields of DHT in response to optimal concentrations of G3, H_2O_2 0.5, H_2O_2G, N250 and H_2O_2GN in human periosteal fibroblasts (HPF) using 14C-testosterone/14C-4-androstenedione as substrate (Figs. 4 and 5).

a. When 14C-testosterone was incubated with periosteal fibroblasts and the above agents, the trends and metabolites formed were similar to those for HGF. The yields of DHT only are shown here (Fig. 4). H_2O_2 reduced yields by 2.3-fold. Addition of G resulted in values similar to controls. Nicotine alone caused a 2.8-fold reduction in yields compared with controls. Addition of N to the neutralized oxidant/antioxidant incubation H_2O_2G reduced yields by 32%. These results were all significant (Fig. 4; $n=6$, $p<0.01$).
b. When 14C-4-androstenedione was used as substrate with HPF, the metabolites formed were DHT, diol and testosterone. The yields of DHT are shown here (Fig. 5), in response to the testing agents. There was a 3-fold reduction in the yield of DHT in response to H_2O_2, which increased by 2-fold when combined with the antioxidant glutathione. Nicotine alone caused a 3–5-fold reduction in yields when compared with controls and

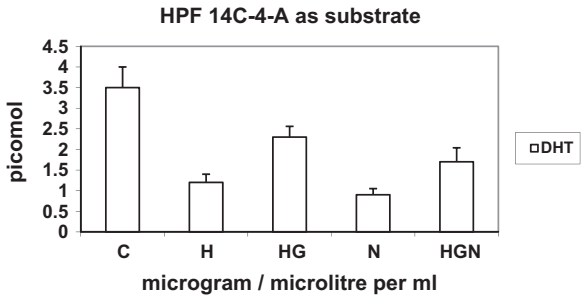

Fig. 5. Yields of DHT in response to optimal concentrations of G3, (H_2O_2) H 0.5, HG, N250 and HGN in human periosteal fibroblasts (HPF) using 14C-4-androstenedione (14C-4-A) as substrate. Yields of DHT from HPF are shown in response to the above agents tested as in Fig. 4, using 14C-4-A as substrate. C: Control, $n=6$; $p<0.01$.

when added to the oxidant/antioxidant incubation, it reduced yields by 26% (Fig. 5; $n=6$, $p<0.01$).

Discussion

The androgen substrates 14C-testosterone and 14C-4-androstenedione were metabolized mainly to diol, DHT and 4-androstenedione or testosterone respectively with the two substrates, in the control incubations as well as in response to testing agents. All metabolites were assayed and yields of the physiologically active androgen DHT are shown in the figures; for the purpose of demonstrating trends in the yields of this biomarker in response to oxidative stress and its amelioration by the antioxidant glutathione. The novel metabolically active *in vitro* model used is effective in reinforcing potential *in vivo* actions, in response to agents tested, using two substrates and cell-types.

Both N and the pure oxidant H_2O_2 alone significantly inhibited yields of DHT. The inhibition caused by H_2O_2 was overcome by the antioxidant glutathione. It is relevant that when nicotine was added to the neutralized combination of H_2O_2G, it reduced the yield DHT to an intermediate value between that of the control and H_2O_2G, demonstrating the oxidant effect of nicotine and at the same time retaining the antioxidant effect of G, on the yield of DHT. This trend is demonstrated across means of 6 incubations each of 4 sets of experiments using HGF and HPF and 2 androgen substrates. The fact that the values for DHT did not revert to control values in the H_2O_2GN incubations confirm the relevance of the antioxidant G in the incubation despite the presence of oxidants N and H_2O_2; thus reinforcing the oxidative actions of N and H_2O_2 and the antioxidant effect of G. There were remarkable similarities between the effects of H_2O_2 and N on the yields of DHT. H_2O_2 is a pure oxidant [15], while N has some oxidative properties; thus confirming oxidative actions of N in the context of our study.

Oral fibroblasts are susceptible to the oxidative effects of stressors such as H_2O_2 and nicotine, which increase ROS; and decrease cell viability and DNA synthesis. These oxidative effects on fibroblasts are overcome in response to polyphenols and antioxidants derived from turmeric, by reducing ROS; and improving cell viability and DNA synthesis. The antioxidant combination used is protective towards fibroblasts in this capacity [21]. A similar study demonstrated that a mixture of antioxidants comprising resveratrol, ferulic acid, phloretin and tetrahydrocurcuminoids counteracted ROS release by metal stressors in oral fibroblasts by increasing cell viability, DNA synthesis and decreasing ROS activity [22].

Other studies show that the cytotoxic apoptotic effects of nicotine and cigarette smoke extract on glioma cells are abolished by *N*-acetyl-L-cycteine and reduced glutathione [23]. A stable component in the cigarette smoke extract is likely to activate bisindolylmaleimide I [BIS I], protein kinase C (PKC) which in turn stimulates NADPH oxidase (NOX) to generate ROS contributing to membrane damage and apoptosis. It was concluded that different ROS are responsible for membrane damage and apoptosis; indicating a role for peroxide and nicotine-mediated systems, using suitable agents. Some apoptotic changes occurred in response to oxidants, independent of NOX and PKC. Similarly glutathione in our combined incubations of H_2O_2GN was able to overcome the oxidative effects of N and H_2O_2 resulting a gradation in values for DHT an effective marker of redox status, demonstrating this effect.

Further mechanisms which account for some of the oxidative effects of nicotine and H_2O_2 have been demonstrated by other workers. There is elevated expression of urokinase-type plasminogen activator receptor (uPAR) during inflammation, tissue modelling and in several human cancers. Nicotine was demonstrated to enhance expression of uPAR in a dose-dependent manner with concurrent activation of the extracellular signal-regulated kinases Erk-1/2, d-Jun amino-terminal kinase (KNK) and p38 mitogen activated protein kinase (MAPK). Specific inhibitors attenuated the nicotine-induced uPAR expression. Intracellular content of H_2O_2 was shown to be elevated in response to nicotine [24]. Nicotine—activated production of ROS and uPAR expression were impaired by the antioxidant acetylcysteine. Exogenous H_2O_2 increased the expression of uPAR mRNA. Binding sites of transcription factor nuclear factor-kappaB (NF-kB) and activator protein (AP)-1 are involved in nicotine-induced uPAR expression; MAPK (Erk-1/2 and JNK) and ROS function as upstream signalling molecules in the activation of AP-1 and NF-kB respectively, indicating that nicotine induces uPAR expression via the MAPK/AP-1 and ROS/NF-kB signalling pathways.

These findings reinforce the results of our study, demonstrating oxidative actions of H_2O_2 and N overcome by glutathione, using DHT as a marker of oxidative stress. The oxidative actions of H_2O_2 and N were demonstrated in staged incubations of H_2O_2, N and H_2O_2G in combination; the combined incubation H_2O_2G with glutathione overcame the oxidative actions of H_2O_2 with raised yields of DHT over H_2O_2 alone; while further addition of N to this combination demonstrated decreased values of DHT demonstrating an oxidative downturn in response to N; but above values for N or H_2O_2 alone indicating antioxidant actions of G being protective in the incubation.

Smokers are also more prone to infections, due to suppression of the immune system by tar products. Of these products, nicotine and other oxidants are inhibitory for sensing bacterial LPS [25]. Stimulation of macrophages with Gram negative bacteria or LPS induces nitric oxide synthase (NOS) and tumor necrosis factor (TNF)-α. Cigarette smoke is found to inhibit NO release by murine macrophages in response to whole bacteria; it also inhibits NO synthase ll protein expression in response to LPS. It is relevant that H_2O_2 mimicked the effects of cigarette smoke extract on LPS-induced nitrite formation which were reversed by the antioxidants *N*-acetyl cysteine and glutathione. It is possible that immunosuppressive effects of cigarette smoke are mediated via oxidative stress-inducing mechanisms. Our *in vitro* study could be extrapolated to an *in vivo* setting in the context of the effects of nicotine addressed here. Specificity of 5α-reductase activity linked to anabolic actions [7] excludes non-specific apoptotic changes. Evidence of wound healing being significantly impaired by oxidants supports this view.

Nicotine signals via nicotinic acetylcholine receptors. It is relevant that in nicotine-treated human dental pulp cells, there is significant reduction in mRNA expression of dentine matrix acidic phosphoprotein-1, bone sialoprotein and alkaline phosphatase activity [26]. In addition, mineralized nodule formation by these cells is also inhibited by nicotine. These findings indicate that nicotine suppresses

cytodifferentiation and mineralization in human dental pulp cells; the mechanism involved is likely to be via nicotinic acetylcholine receptors. In our investigation, inhibition of the yields of DHT in response to nicotine is partly indicative of a reduced capacity for wound healing in the cells tested in view of AR mediated actions of DHT in connective tissue matrices and bone [7,8,10].

Nicotine or LPS demonstrate reduction in alkaline phosphatase activity in osteoblastic cells which decreased further when both were combined in the incubation [27]. Nicotine significantly increases PGE_2 production in response to nicotine, enhanced by LPS and overcome by indomethacin by its actions on PGE formation and AP activity. There is increased phosphorylation of protein kinase A in cells cultured with nicotine and LPS. Nicotine-induced PGE_2 appears to be enhanced by LPS due to increased COX-2 expression. The induced PGE_2 then interacts with an osteoblast receptor in a primarily autocrine or paracrine mode and subsequently decreases ALP activity and increases the expression of macrophage colony stimulating factor; suggestive of the development of a pro-inflammatory phenotype in this environment. In view of a role for nicotine in inducing an inflammatory phenotype, the results of our *in vitro* investigation are particularly relevant to healing potential in the inflammatory milieu of a periodontal pocket in a smoking population.

Impaired wound healing responses to smoking and nicotine have significant effects. Reduced fibroblast migration and proliferation; and associated down-regulation of collagen synthesis contribute to impaired wound healing [28]. This could be explained by an oxidative action affecting alkaline phosphatase activity. There is rapid restoration of tissue oxygenation and metabolism in response to smoking cessation; and partial reversal of the inflammatory cell response within 4 weeks. The findings of our *in vitro* investigation reinforce the oxidative effects of nicotine, which have implications on wound healing by modulating AR-mediated actions relevant to wound healing mechanisms including impairment of alkaline phosphatase activity [27].

Nicotine down-regulates p38 mitogen activated protein kinase (MAPK) phosphorylation and alpha-smooth muscle actin, a specific marker for myofibroblasts and mRNA induced by TGF-beta1 in cultured human gingival fibroblasts [29]; indicative of inhibition of myofibroblast differentiation in human gingival fibroblasts *in vitro*, in response to nicotine and supporting the hypothesis that decreased wound contraction by myofibroblasts could partly account for delayed wound healing in smokers. The results of our investigation indicate that nicotine modulates oxidative stress-induced androgen biomarkers. This could have implications on wound healing responses by decreasing yields of androgen metabolites associated with tissue turnover [7,8,10]. They are suitable biomarkers of wound healing in an environment of oxidative stress.

Several studies have shown that some of the detrimental effects caused by nicotine on diverse metabolic processes could be partly counteracted by antioxidant agents [16,13,12]. These findings suggest that one of the pathways by which nicotine exerts its influence on cell metabolism, is via an oxidative effect, similar to that of other oxidants such as H_2O_2. In our study, the fact that the inhibitory effect of nicotine was at least in part balanced by the presence of glutathione in the combined incubations (H_2O_2GN), is suggestive of an oxidative pathway for the expression of androgen biomarkers in response to nicotine.

Despite the fact that the metabolic profiles did not always show a consistent pattern in response to the agents used, a general trend emerged from our study. These trends were reinforced in replicates using two androgen substrates, in both human gingival and periosteal fibroblasts. Nicotine had some negative effects on androgen metabolism which were significantly similar to those exerted by hydrogen peroxide. This similarity was seen, in the impaired yields of androgen metabolites in response to both nicotine and H_2O_2; and in the way these effects were counteracted by in the presence of glutathione. This would suggest that nicotine exerts a detrimental effect on androgen metabolism via an oxidative mechanism, rather similar to that mediated by H_2O_2. In view of distinct changes seen in response to individual agents and their combinations, these findings exclude the possibility of non-specific apoptotic changes.

Previous literature shows that androgen metabolites play an important role in wound healing responses [7] and that periodontal tissues are important androgen targets [8–10]. Nicotine impairs wound healing in general and the results of the present study seem to indicate that it also impairs androgen metabolizing enzymes and AR- mediated mechanisms in HGF and HPF. These findings could reflect one of several possible mechanisms involved in determining its negative impact on periodontal diseases. Redox status of the environment in which wound healing takes place is an important aspect, since it affects collagen synthesis, demonstrated by other workers.

Postoperative complications of wound healing are more prevalent in smokers than in non-smokers. Impaired wound healing and connective tissue turnover are suggested mechanisms [30]. Examination of punch biopsy wounds in smokers graded as continuous smoking; abstinence with transdermal nicotine patch; abstinence with placebo patch; and 30 never smokers, demonstrated that smokers have smaller, superficial wounds with reduced serum levels of vitamin C and procollagen 1 N-propeptide. These findings are suggestive of altered wound healing parameters in response to smoking associated with vitamin C levels, collagen turnover and inflammatory responses. Nicotine has proven oxidative effects on tissues exposed to it [25,14]. It is relevant that results from our *in vitro* investigation reinforce some of these findings. Glutamine would function in a similar mode to vitamin C in this context, in an antioxidant capacity.

Constituents of cigarette smoke including nicotine contribute to detrimental effects on cell metabolism. Identification of up-regulation or down-regulation of differentially expressed genes in macrophage-like human cell lines in response to nicotine, indicates that nicotine enhances host inflammatory responses and alters defence mechanisms against pathogens [31]. The response of HGF and HPF to nicotine, in our *in vitro* study comprising an oxidative stress-inducing environment; and responses to the antioxidant glutathione, are suggestive of its importance in maintaining an inflammatory milieu in the periodontal lesion. This could affect treatment responses.

Besides, the anabolic effect exerted by androgens [32] seem to require an environment that is relatively free of oxidants. It is relevant that these hormones induce the activation of genes for antioxidant enzymes [7]. Steroid hormones activate signal transducers that activate a range of cellular responses. Testosterone has been identified as an antioxidant amongst these hormones, being protective against cellular damage. Using a mouse embryonic stem cell model, the biologically active testosterone metabolite dihydrotestosterone (DHT) was evaluated for its effects on H_2O_2-mediated apoptosis, reduced cell viability, reduced DNA synthesis and cell cycle regulatory protein synthesis. [33]. Pretreatment with DHT, inhibited these effects by reducing intracellular levels of HO; which were abolished by the androgen receptor inhibitor flutamide. The presence of androgen receptor in mouse embryonic stem cells supported this activity. Increased levels of p38 MAPK, JNK/SAPK and NF-kappaB phosphorylation induced by H_2O_2 were inhibited by pretreatment with DHT. These effects were also inhibited by the antioxidant enzyme catalase enhanced by DHT; while flutamide abolished these inhibitory effects.

These findings indicate that DHT prevents H_2O_2-induced apoptotic cell death of mouse embryonic stem cells via androgen receptor-associated activation of catalase and downregulation of p38 MAPK, JNK/SAPK and NF-kappaB. These effects could be

extrapolated to the results of our investigation using DHT as a biomarker for the effects of H_2O_2 on HGF and HPF, confirming the actions of N in this capacity.

The enzyme systems involved also seem to have direct antioxidant effects themselves [6,34]. In our study, the possible pro-oxidant effect of nicotine exerted on expression of the androgen biomarker DHT was tested against that of H_2O_2, the most common oxidant naturally produced by physiological activities of cells. Due to acute toxicity of H_2O_2, scavenging enzymes elaborated by bacteria maintain its intracellular concentrations at nanomolar levels. Primary scavengers in many bacteria are catalases and NADPH peroxidase; other enzymes with a similar role include thiol peroxidase, glutathione peroxidase, cytochrome c peroxidase and rubrerythrins. The biochemical, regulatory, genomic and genetic evidence of cellular radical scavenging actions of these enzymes have been reviewed [35]. There is a need for multiple bacterial enzymes in order to catalyse this process, which include diverse substrate specificities, cofactor requirements, kinetic optima and enzyme stability. Clarification of these mechanisms could contribute to a better understanding of features of oxidative stress and stress resistance. As an antioxidant capable of overcoming the effects of both H_2O_2 and nicotine, namely glutathione was chosen for its specific physiological role and relevance to inflammatory repair.

Alkaline phosphatase activity is reported to be influenced by the redox status of the environment. Modulation of low molecular weight protein tyrosine phosphatases a group of enzymes associated with cell growth and differentiation are shown to be coincident with osteoblastic differentiation markers such as alkaline phosphatase (AP) and the presence of mineralizing bone nodules *in vitro* [36]. Similarly, the reduced glutathione-dependent microenvironment is modulated in response to redox status of cells associated with suitable levels of reduced glutathione during osteoblastic differentiation; being an important signalling molecule. H_2O_2-induced oxidative stress has been shown to demonstrate an attenuated antioxidant enzyme profile comprising catalase, glutathione S-transferase, glutathione peroxidase and superoxide dismutase in differentiated osteoblasts [37]. AR-mediated responses to agents tested in our *in vitro* study reinforce the importance of redox status in wound healing.

AP has shown to be inhibited in the presence of nicotine [26]. Androgen substrates incubated with fibroblasts demonstrate increased yields of redox markers of wound healing in response to AP. This response is impaired by nicotine and the AP inhibitor levamisole [38]; indicating a possible link between antioxidant effects and AP-mediated actions. It is relevant that in our investigation, glutathione was effective in overcoming the oxidative effects of nicotine and H_2O_2, using steroid biomarkers which are mediated by the functions of androgen receptor, including modulation of AP activity and matrix synthesis. Therapeutic effects of simvastatin on cell viability, apoptosis, and alkaline phosphatase activity in murine osteoblastic cells have been demonstrated in response to H_2O_2 [39]. Simvastatin suppressed H_2O_2-induced oxidative stress and attenuated H_2O_2-induced cell injury including increased osteoblastic viability, inhibition of apoptosis, and enhanced cell- differentiation. Some of these antioxidant effects of simvastatin are comparable to those of glutathione in our investigation.

PMN function may be affected differentially by cigarette smoking during periodontitis; affecting the elimination of periodontal pathogens effectively while in heavy smokers ROS release and tissue damage mediated by oxidative stress may be relevant [40]. Inhibition of translational activity conserves energy for repair of cell damage in response to stress. Accumulated untranslated mRNAs in these cells move to discrete cytoplasmic foci known as stress granules (SGs). These SGs assist cells in surviving adverse environmental conditions. H_2O_2 appears to inhibit translation and induce the assembly of SGs [41], demonstrating novel aspects of translational regulation in response to oxidative stress. Some of these mechanisms and findings of our study regarding the actions of nicotine and H_2O_2 may be extrapolated to the redox environment in an inflammatory periodontal lesion in smokers; with the effects of glutathione akin to treatment responses on removal of inflammatory stimuli.

Conclusions

These findings provide insight into antioxidant enzyme activity and redox pathways mediated by AR which may be extrapolated from our results by virtue of mechanism of actions of androgens via AR. This could influence vulnerability of cells used in our *in vitro* cultures, to oxidative stress. These mechanisms could explain pro-oxidant effects of nicotine with relevance to periodontal wound healing in an inflammatory environment. A novel metabolically active model has been used, using two androgen substrates and two critical cell-types yielding the marker DHT, relevant to redox healing; demonstration of metabolic yields of DHT in response to agents tested enables closer extrapolation to *in vivo* applications.

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