The mitogenic effect of H_2O_2 for vascular smooth muscle cells is mediated by an increase of the affinity of basic fibroblast growth factor for its receptor

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Abstract Increased generation of active oxygen species such as hydrogen peroxide (H_2O_2) may be important in vascular smooth muscle cell growth associated with atherosclerosis and restenosis. In this work, we showed that H_2O_2 was a potent mitogen for growth-arrested cultured human aortic smooth muscle cells (SMC), stimulating an increase in cell number at 10 nM to 100 μM concentration. This effect was inhibited in a dose-dependent manner by catalase, deferoxamine, dimethylthiourea or probucol showing that it was dependent on the oxidative activity of H_2O_2. H_2O_2-induced SMC proliferation was strongly and specifically inhibited by a neutralizing monoclonal antibody directed against basic fibroblast growth factor (bFGF) but was not due to increased expression of bFGF or the bFGF receptor-1 (FGFR-1) by SMC. H_2O_2 strongly increased the affinity of bFGF for its receptor-1 at the surface of the SMC, therefore showing that the mitogenic effect of H_2O_2 might occur through a direct effect on the bFGF receptor.

Key words: Smooth muscle cell; Proliferation; Hydrogen peroxide; H_2O_2; Basic fibroblast growth factor

1. Introduction

The accumulation of neointimal smooth muscle cells (SMC) resulting from media smooth muscle proliferation and migration in response to vascular injury is believed to be one of the main events involved in the initiation of atherosclerosis [1,2]. Nevertheless, the mechanism by which various types of growth factors are secreted by platelets, macrophages or vascular cells to initiate and sustain the replication of vascular SMC leading to the formation of neointima after vascular injury is poorly understood.

Although the general contribution of growth factors and cytokines in the development of atherosclerosis has been acknowledged for a long time, recent investigations suggest that oxidants, since they have been shown to activate a number of pathways that are also stimulated by growth factors, can be considered as early growth signals [3]. In particular, oxidants can induce transcription of enzymes, such as ornithine decarboxylase [4], phosphatase CL-100 [5] and of proto-oncogenes [6–9]. It has also been suggested that oxidants enhance the activity of protein kinase C (PKC) [10–13], that of the transcriptional activator NF-κB and AP-1 [14] and activate MAP kinase in SMCs [15].

Recent reports have also shown that hydrogen peroxide (H_2O_2) stimulates DNA synthesis in SMC [6,16] and induces the expression of several growth-related proto-oncogenes, including c-myc and c-fos [6]. Other investigators have also demonstrated that H_2O_2 and O_2^- stimulate growth-related events such as cell alkalinization and proto-oncogene induction [17,18] and it has been recently demonstrated that H_2O_2 was required as a signal transducing molecule for platelet derived growth factor (PDGF) to stimulate the growth of vascular SMCs [19]. In addition, evidence indicates that experimental atherosclerosis, restenosis and foam cell formation can be effectively retarded by antioxidants [20–24] and the administration of antioxidants to human subjects has been shown to increase the resistance of their low density lipoproteins to oxidation and to protect against atherosclerosis [25–28].

In view of these data, the present study was undertaken to gain an insight into the mechanism by which the oxidant H_2O_2 stimulates the growth of human aortic SMCs in culture and to test the possible involvement of growth factors in this phenomenon.

2. Materials and methods

2.1. Materials

H_2O_2, catalase, probucol and dimethylthiourea (DMTU) were obtained from Sigma Chemical Co. (L'Isle d'Abeau, France). Deferoxamine (DFO) was obtained from Ciba (Summit, NJ, USA). All culture reagents were purchased from Boehringer Mannheim (Mannheim, Germany). Monoclonal neutralizing antibodies against human bFGF (Ab-bFGF), PDGF-BB (Ab-PDGF), aFGF (Ab-aFGF), TGFβ (Ab-TGF) and EGF (Ab-EGF) were obtained from R&D Systems (Minneapolis, MN).

2.2. Culture and proliferation assays of human vascular smooth muscle cells

Human aortic smooth muscle cells (ASMC) were purchased from Clonetics (Le Perray, France). These cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml of penicillin, 50 μg/ml of streptomycin sulfate and 4 mM glutamine (Boehringer-Mannheim, France). For measurement of cell growth, ASMC were seeded in DMEM+0.5% FCS at 1×10^4 cells per well (24-well plates, Falcon Beckton Dickinson, Le Pont de Claix, France) for 3 days in a humidified CO_2 incubator maintained at 37°C. Culture medium was then removed and cells were seeded in DMEM+0.5% FCS with various concentrations of H_2O_2. The inhibition of proliferation induced by H_2O_2 (0.1 mM) by various compounds was also studied under the same experimental conditions. For growth rate determination, after 3 days in culture, triplicate plates were trypsinised and the cells counted with a Coulter counter (Coultronics, France).

For binding experiments, ASMC were seeded in 24-well cluster plates (3×10^4 cells/well) in DMEM+10% FCS and grown to confluence (4–5 days after plating) ($2–3\times10^5$ cells/well). Cells were routinely used between the third and 10th passage.

2.3. ^{125}I-bFGF binding to ASMC

Experiments studying the specific binding of ^{125}I-bFGF (110 μCi/μg) (NEN Research Products, Dupont, France) to human ASMC

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were performed on cells cultured in 24-well cluster plates. Subconfluent cultures ($2–3\times10^5$ cells/well) were incubated at 4°C for 10 min and washed twice with ice-cold DMEM supplemented with 25 mM HEPES (pH 7.4), 0.3 mg/ml soybean trypsin inhibitor (STI), 0.5 mg/ml bacitracin and 0.2% gelatin (Sigma Chemical Co., L'Isle d'Abeau, France). Incubations were carried out in a total 200 µl volume of HEPES 25 mM (pH 7.4) supplemented with 0.3 mg/ml STI, 0.5 mg/ml bacitracin and 0.2% gelatin which contained different concentrations of ^{125}I-bFGF. Triplicate experiments were carried out at 7°C for the indicated periods of time. To determine the total binding of ^{125}I-bFGF, the cells were incubated for 5 min in 1 ml of 1 M NaOH and the extract was counted in a gamma counter. Non-specific binding was considered as the value obtained for high affinity binding in the presence of a 100-fold excess on non-labeled bFGF (Amersham; Les Ulis, France). In all experiments, representative wells were trysinised and cells were counted with a Coulter counter.

2.4. Expression of bFGF and FGFR-1 mRNA

Total cellular RNA was isolated from 5×10^6 cells according to Chomczynski and Sacchi [29] and bFGF and FGFR-1 mRNA expression was analyzed by Northern blotting. Samples were size-fractionated by electrophoresis on 1% agarose gels containing 7.5% formaldehyde. Capillary transfer to Nylon N^+ (Amersham, UK) was performed overnight in standard saline citrate (SSC). Hybridizations were carried out at 42°C for 18 h in hybridization buffer (50% formamide, 1 M NaCl, 10× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 100 µg/ml salmon sperm DNA). DNA probes used for Northern blot hybridization analysis were plasmid pJJ1-1 [30] and clone pCD115 [31] for bFGF and FGFR-1, respectively. Washes were performed at room temperature in 2×SSC, 0.1% SDS and finally at 60°C in 0.1×SSC, 0.1% SDS. Filters were rehybridized with a probe for human GAPDH as a control for the amount of RNA in each lane and washed under the same conditions as above. Autoradiograms were obtained by exposure of filters to Amersham MP films at −80°C using two intensifying screens. Relative intensities of hybridization signals were obtained by densitometer scanning of autoradiograms (Vilbert Lourmat, France).

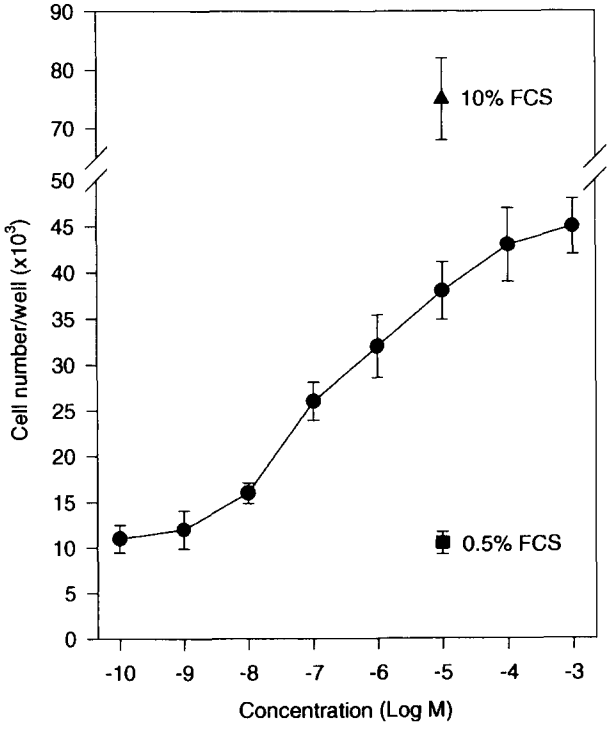

Fig. 1. Mitogenic effect of H_2O_2 for human aortic SMCs. Human SMCs were plated sparsely (10^4 cells/well) in 24-well cluster plates in DMEM + 0.5% FCS. After 3 days, growth-arrested cells were exposed to increasing concentrations of H_2O_2 (●), 0.5% FCS (■) or 10% FCS (▲). After 3 days, cells were detached from triplicate wells by trypsin treatment and counted in a coulter counter. Results are expressed as mean cell numbers ± SD ($n = 6$).

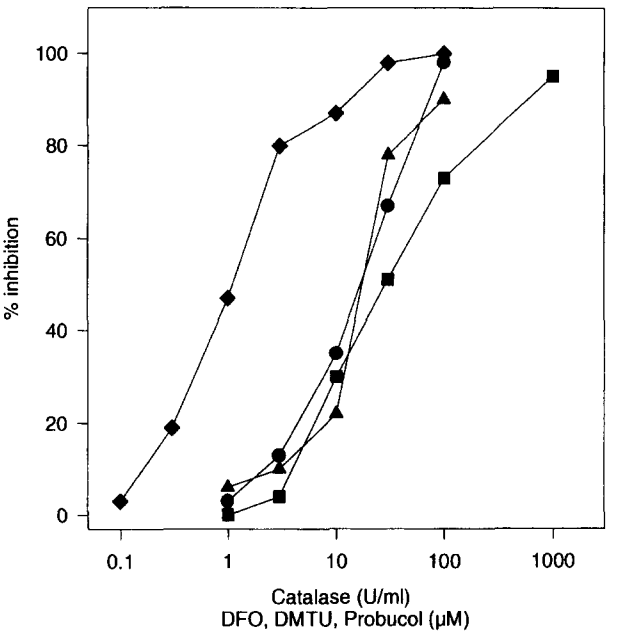

Fig. 2. Effect of various compounds on the mitogenic activity of H_2O_2. Human SMCs (10^4 cells/well) were cultured in 24-well cluster plates in DMEM+0.5% FCS. After 3 days, H_2O_2 (0.1 mM) was added in the presence of increasing concentrations of catalase (●), deferoxamine (▲), dimethylthiourea (■) or probucol (◆). After 3 days in culture, cells were detached from triplicate wells and counted. Results are expressed as percent inhibition of the cell proliferation measured in the vehicle-treated wells.

2.5. Expression of data

All data are means of at least triplicate experiments. The apparent dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were calculated by using Scatchard representation of the experimental data [32]. Data from saturation studies were analyzed using a non-linear regression program [33].

3. Results and discussion

3.1. Mitogenic effect of H_2O_2 on human vascular SMC

Although vascular SMC hyperplasia is now considered as a key event in atherogenesis and restenosis, specific regulatory mechanisms for cell proliferation in the arterial wall are still poorly characterized. While in vitro studies with vascular SMCs have identified several agents that stimulate their proliferation in culture, growth factors responsible for vascular injury-induced intimal SMC replication have only begun to be identified. Along with PDGF [34], bFGF [35] and several other growth factors and cytokines that are now considered to be likely candidates to play an important role in the formation of neointima following endothelial damage, oxidants, since they have been shown to activate a number of pathways that are also stimulated by growth factors, can be considered early growth signals [3].

The molecular mechanisms associated with cell injuries caused by oxidants are not completely understood but several works suggest that H_2O_2 might increase arachidonic acid release by stimulating membrane-associated phospholipase A_2 (PLA2) activity [36,37], increase the activity of protein kinases

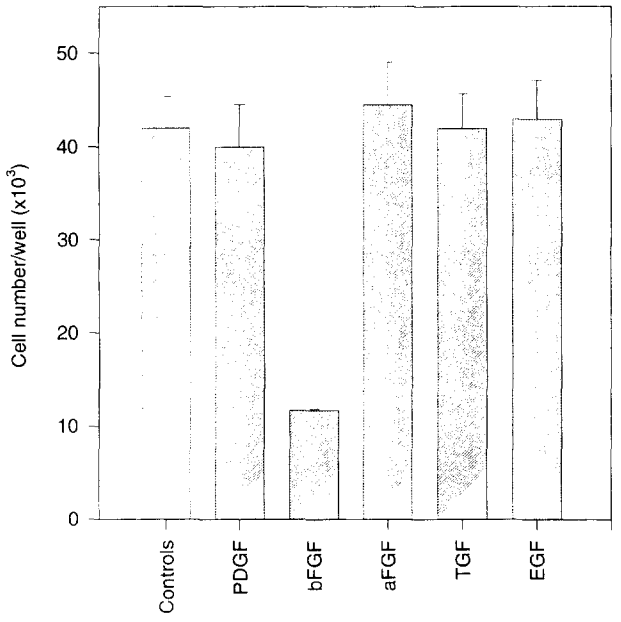

Fig. 3. Effect of various antibodies on the mitogenic activity of H_2O_2. SMCs (10^4 cells/well) were cultured in 24-well cluster plates in DMEM + 0.5% FCS. After 3 days, H_2O_2 (0.1 mM) was added in the presence of 0.5% FCS and the indicated antibodies (100 μg/ml). After 3 days in culture, cells were detached from triplicate wells and counted. Results are expressed as mean cell numbers ± SD ($n = 6$).

and phosphatases in vascular SMCs [15], activate early response genes [6–9] and induce DNA synthesis in growth-arrested cells in vitro [6,13,15,16,19] or in vivo following arterial injury [24]. On the basis of these findings, we hypothesized that active oxygen species should activate SMC growth via a particular growth factor as already shown for numerous other substances such as thrombin [38], tissue plasminogen activator [39] or hypoxia [40].

In the present study, we demonstrate that, when added to growth-arrested human SMCs, H_2O_2 exhibited a dose-dependent mitogenic effect (Fig. 1). This activity was significant from the dose of 10 nM and reached a maximum between 0.1 and 1 mM. At these concentrations, H_2O_2 induced a 4.5-fold increase in cell number over a period of 3 days. This effect represented about 60% of the mitogenic effect of 10% FCS. The concentration of H_2O_2 required to obtain a half-maximal response (ED_{50}) was of 0.2 ± 0.04 μM ($n = 6$). As shown in Fig. 2, the mitogenic effect of H_2O_2 was closely related to its oxidative activity since catalase and probucol inhibited in a dose-dependent manner H_2O_2-induced proliferation with IC_{50} values of 20 ± 4 U/ml and 1.2 ± 0.2 μM ($n = 6$). It is noteworthy that these concentrations corresponded closely to the inhibitory effect of catalase and probucol with regard to the oxidative activity of H_2O_2 as measured in the incubation medium (IC_{50} = 17 ± 5 U/ml and 1.0 ± 0.3 μM $n = 6$). The specific iron chelator DFO has been shown to modify tissue changes observed in oxidant injury [41,42] and DFO is widely used as a probe to study iron-dependent radical(s) production [41]. Our present study indicates that pretreatment with DFO markedly reduced H_2O_2-induced SMC growth (Fig. 2). DMTU, a potent $OH^{\bullet}$ scavenger [43] also strongly inhibited the mitogenic effect of H_2O_2 therefore demonstrating the importance of this radical, as already shown on these same cells for other effects of H_2O_2 [37]. Collectively, these results suggest that the mitogenic effect of H_2O_2 occurs predominantly via OH^-, the formation of which has been shown to occur with iron as a catalyst [44]. Stimulation of arachidonic acid release from pulmonary SMC by H_2O_2 has been shown to occur via the same mechanism [37]. Moreover, these results might provide an explanation for the antiproliferative activity of probucol, a compound which has been shown to decrease the myointimal proliferation of injured vessels in animals receiving a cholesterol-rich diet [22,23].

In order to determine if such a mitogenic effect of H_2O_2 for SMCs could be due to the release of growth factors by the cells, we evaluated the activity of neutralizing monoclonal antibodies directed against PDGF-BB, bFGF, aFGF, TGFβ and EGF. Only the monoclonal antibody directed against bFGF inhibited H_2O_2-induced growth of SMC (Fig. 3). This inhibitory effect did not occur with regard to fetal calf serum-induced SMC growth (not shown) and was not a general phenomenon involving other growth factors since we observed no significant inhibitory effect of the monoclonal antibodies directed against the other growth factors with regard to the mitogenic effect of H_2O_2.

3.2. *Effect of H_2O_2 on bFGF and FGFR-1 expression in human SMCs*

Since our results demonstrate for the first time that H_2O_2-induced SMC growth might occur via bFGF as an essential intermediary, we determined whether H_2O_2 could affect the expression of bFGF mRNA in SMC but, upon stimulation with H_2O_2, no significant rise in bFGF mRNA expression was observed within SMCs (Fig. 4). A similar result was obtained with regard to the expression of the FGF receptor type 1 (FGFR-1), the only type of receptor present on SMC [45], therefore showing that the mechanism of the mitogenic action of H_2O_2 was not at the level of the bFGF or FGFR-1 mRNA transcription.

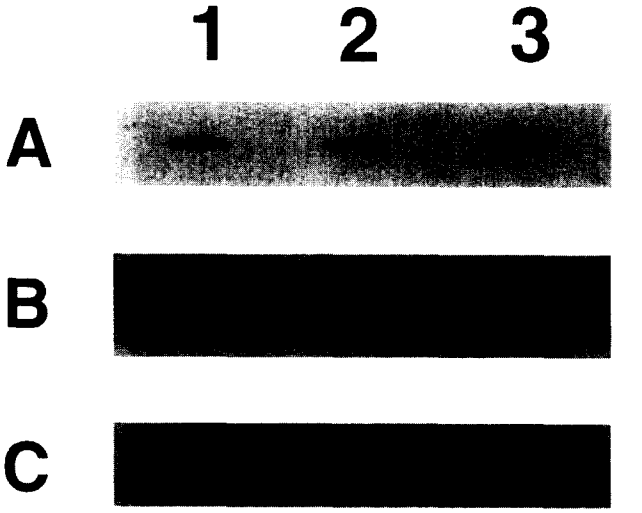

Fig. 4. Effect of H_2O_2 on the expression of bFGF and FGFR-1 mRNA in SMCs. Growth-arrested SMCs (10^6 cells) were incubated for 3 days with saline (lane 1), H_2O_2 (0.1 mM) (lane 2) or 10% FCS (lane 3). Total mRNA was purified and Northern blot analysis was performed by using cDNA probes for bFGF (A) or FGFR-1 (B) described under Section 2. GAPDH (C) was used to normalize RNA loading. Results are representative of 3 experiments.

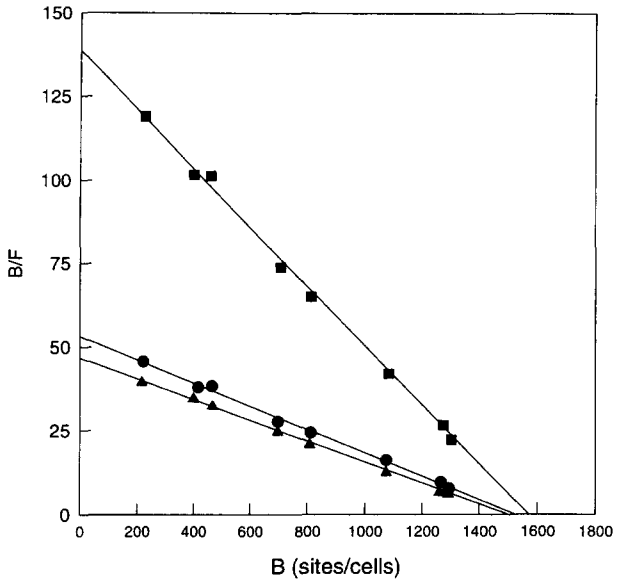

Fig. 5. Effect of H_2O_2 on the binding of ^{125}I-bFGF to HASMC. Growth-arrested SMC (3×10^5 cells/well) were incubated for 3 days with saline (●), H_2O_2 (0.1 mM) (■) or H_2O_2 + catalase (0.1 mM + 100 U/ml) (▲). SMC were then rinsed and incubated for 3 h at 7°C with increasing concentrations of ^{125}I-bFGF (0–140 pM). Specific binding was given by the difference between total and non-specific binding determined in the presence of 7.5 nM of unlabeled bFGF. Scatchard plots of the specific binding of ^{125}I-bFGF was calculated from saturation isotherms determined from at least 3 independent experiments performed in triplicate.

3.3. Effect of H_2O_2 on ^{125}I-bFGF binding to human SMCs

To investigate further how bFGF might modulate the mitogenic effect of H_2O_2, we determined its effect on the binding of ^{125}I-bFGF to HASMC. As shown in Fig. 5, ^{125}I-bFGF bound to a single class of saturable receptor sites. This site showed a high affinity for ^{125}I-bFGF ($K_d = 90 \pm 12$ pM) and a binding capacity (B_{max}) of 1540 ± 12 sites/cell. Following treatment of HASMC with H_2O_2, the affinity of these sites for ^{125}I-bFGF increased 3.5-fold whereas the binding capacity was not affected. The addition of catalase to H_2O_2 normalized the binding characteristics of ^{125}I-bFGF to HASMC, therefore showing that the effect of H_2O_2 on ^{125}I-bFGF binding was indeed due to its oxidative activity. Although the mitogenic effect of several substances has already been shown to be dependent on the induced expression and release of bFGF by SMC [38,39], no other studies have demonstrated the induced increased of its affinity for the receptor present on SMC. The etiology of this activity of H_2O_2 remains unknown but one can postulate that direct oxidation of critical protein sulfhydryl groups may be responsible for such an effect. Examples of active oxygen species acting in this fashion include dimerization of Fos-Jun proteins [46], activation of NF-κB [47], activation of endoplasmic reticulum tyrosine kinases [48] and stimulation of kinase-involved in growth-related signal transduction [49]. In these respects, these previous studies indicate that active oxygen species share properties with growth factors. However, a direct effect of H_2O_2 on bFGF (exogenous or produced endogenously) might not explain this increase of affinity. Indeed, bFGF has 2 reduced cysteine residues that may be targets for oxidizing effect of H_2O_2 but several studies have shown that chemical treatments that result in modification of cysteine residues in bFGF inactivate the growth factor [50,51].

In summary, our present results suggest that: (i) H_2O_2 caused stimulation of SMC proliferation predominantly via its oxidative activity and in particular via OH$^\bullet$ and iron; (ii) H_2O_2-induced SMC occurs via bFGF as an essential intermediary, an increase of the affinity of bFGF for its type-1 receptor being primarily responsible for the mitogenic activity of H_2O_2 for HASMC.

Moreover, although these results suggest that H_2O_2 may act as a potent factor in the development of atherosclerosis and offer a rational explanation for the mechanism of the cardioprotective effect of antioxidants, the exact mechanism by which H_2O_2 directly affects FGFR-1 will have to be determined precisely.

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