Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H_2O_2 generation in 3T3 L1-cells

Heidemarie I. KRIEGER-BRAUER and Horst KATHER

Klinisches Institut für Herzinfarktforschung an der Medizinischen Universitätsklinik Heidelberg, Bergheimerstrasse 58, 69115 Heidelberg, Germany

3T3 L1-cells, which undergo adipose conversion *in vitro*, possess a stimulus-sensitive H_2O_2 -generating system in their plasma membrane, and its properties are virtually identical with those of the insulin-sensitive human fat-cell oxidase [Krieger-Brauer and Kather (1992) *J. Clin. Invest.* **89**, 1006–1013]. Insulin and insulin-like growth factor I were found to be active stimulators of NADPH-dependent H_2O_2 generation. Surprisingly, the acidic (a) and basic (b) isoforms of fibroblast growth factor (FGF) as well as the AA and BB homodimers of platelet-derived growth factor (PDGF) had antagonistic effects on NADPH-dependent H_2O_2 generation in plasma membranes which were paralleled by corresponding changes in H_2O_2 accumulation in intact cells. bFGF and PDGF BB (which inhibit NADPH-dependent H_2O_2

generation) prevented the adipose conversion of 3T3 L1-pre-adipocytes, and this effect could be reversed by exogenously supplied H_2O_2 . Conversely, aFGF and PDGF AA, which stimulated H_2O_2 generation, accelerated adipocyte conversion in the presence of insulin and were adipogenic in themselves. Consistently, expression of the adipocyte phenotype induced by insulin, dexamethasone and isobutylmethylxanthine was enhanced in the presence of exogenous hypoxanthine/xanthine oxidase, whereas antioxidants, such as *N*-acetylcysteine or ascorbate, suppressed the process of differentiation. It is concluded that the H_2O_2 produced in response to hormones and cytokines may contribute to the development and maintenance of the differentiated state.

INTRODUCTION

In a foregoing report we have shown that mature human adipocytes possess a plasma membrane-bound H_2O_2 -generating system that is under antagonistic control by a large and diverse group of hormones and cytokines which typically act through several distinct receptor families [1]. The complex regulation of this plasma-membrane redox activity suggested a fundamental role for H_2O_2 in normal fat-cell physiology. Indeed, H_2O_2 and oxygen radicals qualify as second messengers and may be involved in the regulation of cell growth and differentiation [1–3]. We therefore hypothesized that the H_2O_2 produced in response to hormones and cytokines by adipocytes might contribute to the regulation of fat-cell size and maintenance of the differentiated state. Unfortunately, the limited viability of human adipocytes in primary culture hindered efforts to test this hypothesis directly.

3T3 L1-preadipocytes are fibroblastic cells destined for adipocyte lineage which are widely used to study the mechanisms by which hormones and environmental signals control cell differentiation [4–9]. Consistent and uniform conversion into adipocytes is obtained when postconfluent 3T3 L1-cells are treated for 48 h in fetal calf serum-containing medium supplemented with dexamethasone, isobutylmethylxanthine (IBMX) and high concentrations of insulin [5]. Several cytokines, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor β , have been shown to be antiadipogenic [6–9]. It should be noted that the activity of each of these cytokines is due to a family of closely related peptides with similar biological activities. For example,

PDGF is a disulphide-linked dimer of two related polypeptide chains, designated A and B, which are assembled as heterodimers (PDGF AB) or homodimers (PDGF AA and PDGF BB). PDGF BB and PDGF AB interact with two distinct PDGF receptors (termed α and β) of similar size, whereas PDGF AA binds to α -receptors only [10]. The FGF family consists of nine related proteins [11]. Two distinct types of cell-surface receptors for FGF family members have been identified. These include four tyrosine kinase FGF receptors and a family of heparan sulphate proteoglycans [12,13]. FGF binding and signalling appear to require a co-operative interaction of both types of receptors. Although the receptor kinases for FGFs in conjunction with heparan sulphate proteoglycans clearly participate in the mediation of some of the biological activities of FGF, there is mounting evidence to suggest that these receptors may not be sufficient for all aspects of FGF signalling. First, basic (b)FGF (FGF2) and int-2 (FGF3) are also translated as longer forms starting from CUG codons upstream from the normal initiation codon [13,14]. These isoforms have been found in the nuclei of cells, suggesting that FGFs may also possess an intracellular mode of action. Indeed, recent evidence suggests that bFGF can regulate transcription directly in the nucleus in a gene-specific manner [15]. Secondly, most recently a third type of FGF receptor has been identified that has no known homologies to other proteins, exhibits a 50–60-amino acid cysteine-rich repeated motif, and has an extremely short cytoplasmic domain without inherent catalytic activity [16].

Despite the apparent complexity of the mechanisms involved in the transmission of their signals, the biological effects of

different isoforms of FGF and PDGF are remarkably similar in most cell types that have been studied [10–13]. It was therefore surprising that PDGF AA and PDGF BB as well as the acidic (a) and basic isoforms of FGF had antagonistic effects on NADPH-dependent H_2O_2 generation in human fat-cell ghosts [2]. In this report it is shown that these same isoforms of PDGF and FGF have analogous effects in 3T3 L1-cells, and evidence is provided to suggest that the stimulus-sensitive plasma-membrane redox activity of 3T3 L1-cells is important to the regulation of adipocyte differentiation.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM) with 25 mM glucose, xanthine oxidase and insulin were purchased from Boehringer-Mannheim (Mannheim, Germany). Fetal calf serum (FCS) and dexamethasone, as well as FGF and PDGF isoforms, were obtained from Sigma (München, Germany).

Cell culture and differentiation

3T3 L1-preadipocytes (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in high-glucose DMEM containing 10 % FCS, 2 mM L-glutamine, 100 units/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin, which was changed every second day.

At confluence (day 0), the medium was changed for DMEM supplemented with 5 % FCS that had been heat-inactivated for 45 min at 56 °C [7,8]. Differentiation was routinely induced with 10 nM insulin, 0.5 μM dexamethasone and 0.5 mM IBMX for 48 h [5]. Thereafter cells were treated with 10 nM insulin and 5 % heat-inactivated FCS every second day. When added, cytokines [insulin-like growth factor I (IGF-I), PDGF and FGF], antioxidants (*N*-acetylcysteine and ascorbic acid) or exogenous hypoxanthine/xanthine oxidase were present during the entire course of the differentiation protocol.

Preliminary experiments showed that the generation of reactive oxygen species and the effects of antioxidants varied with different batches of xanthine oxidase and FCS. Therefore a single lot of xanthine oxidase and the same batch of serum were used for all experiments.

Assessment of differentiation

The extent of differentiation was quantified by staining accumulated lipid with Oil Red O after fixation of the cells with 5 % formaldehyde, and by measuring cellular triacylglycerol content and glycerol-3-phosphate dehydrogenase (GPDH) activity.

For analytical procedures, the cells were washed with isotonic NaCl, and then harvested into 0.3–0.5 ml of 25 mM Tris/HCl/1 mM EDTA, pH 7.5. The cells were sonically disintegrated for 3 s at 30 W. Assay of GPDH was carried out by measuring the reduction of NAD via sequential conversion of glycerol 3-phosphate into glycerate 3-phosphate, which is irreversible in the presence of arsenate [17]. The assay mixture contained 14 mM triethanolamine, pH 9.0, 0.5 mM KH_2PO_4 , 10 mM Na_3AsO_4 , 30 mM dithiothreitol, 1.4 mM MgCl_2 , 4 mM NAD^+ , 3 mM glycerol 3-phosphate, 9.5 units/ml glyceraldehyde-3-phosphate dehydrogenase and 17 units/ml triose phosphate isomerase. Reactions were started by addition of appropriate aliquots of the homogenates, and were continued for 30 min at 37 °C. NADH formation was monitored using the bacterial NAD(P)H-linked luciferase [17]. The specific activity of GPDH

was expressed in m-units (nmol of NADH/min) per mg of protein. Cellular triacylglycerol content was determined using a commercially available test combination (Human, Taunusstein, Germany). Protein content of the homogenates was measured by the Lowry procedure after precipitation in 10 % (w/v) trichloroacetic acid [18].

H_2O_2 release by intact 3T3 L1-cells

Confluent cultures of 3T3 L1-cells were incubated in PBS supplemented with 1.2 mM CaCl_2 , 1.4 mM MgCl_2 , 10 mM glucose, 1 mM NaN_3 and 0.1 % human serum albumin in the absence or presence of insulin and/or cytokines, as indicated. After 10 min, aliquots of the media were removed and assayed for H_2O_2 [1].

Preparation of preadipocyte plasma membranes

Confluent 3T3 L1-cells were incubated in DMEM containing 0.1 % human serum albumin in the absence of hormones or cytokines for 24 h before plasma-membrane preparation. Thereafter the medium was decanted and cells were washed in PBS. The cells were broken open by freeze-thawing. The resulting lawn of plasma-membrane fragments was repeatedly washed with, and then scraped into, a hypotonic medium containing 20 mM Mes, pH 5.8, 2 mM MgCl_2 , 1 mM CaCl_2 , 5 mM KCl and 100 mg/l soya-bean trypsin inhibitor. As shown by others [19], this technique yielded a highly purified plasma-membrane fraction.

Receptor-mediated modulation of NADPH-dependent H_2O_2 generation

A two-step procedure was used as reported elsewhere [1]. Plasma membranes were first exposed to ligands and Mn^{2+} (activation step), and were then assayed for NADPH oxidase activity. The activation step was carried out in 30 mM Mops, pH 7.5, containing 120 mM NaCl, 1.4 mM CaCl_2 and 0.1 % human serum albumin. Membranes were first incubated with various ligands for 5 min to allow the formation of ligand–receptor complexes. Thereafter 3 mM MnCl_2 was added and preincubations were continued for another 25 min. After 30 min ghosts were collected by centrifugation, washed and then resuspended in 30 mM Mes, pH 5.8, containing 120 mM NaCl, 4 mM MgCl_2 , 1.2 mM KH_2PO_4 , 1 mM NaN_3 and 10 μM FAD.

NADPH oxidase assays were started by addition of 0.25 mM NADPH and were terminated after 30 min at 37 °C by adding HCl (H_2O_2 determinations) or KOH (NADPH measurements) to yield final concentrations of 0.1 mM. H_2O_2 was determined as described [1]. Assays were routinely performed at four different protein concentrations ranging from 2 to 20 $\mu\text{g}/\text{ml}$ protein.

RESULTS

Antagonistic effects of different members of the PDGF and FGF families on NADPH-dependent H_2O_2 generation in crude plasma membranes and in intact 3T3 L1-cells

3T3 L1-preadipocytes (and adipocytes) contained a stimulus-sensitive redox activity, producing 1 mol H_2O_2 /mol of NADPH oxidation, that was localized to the plasma-membrane fraction (Table 1). Its properties (pH optimum, substrate affinity, stoichiometry between NADPH oxidation and H_2O_2 production, stability of regulation) closely resembled those of the human fat-cell oxidase [1,2]. Most importantly, the system of 3T3 L1-cells also retained its sensitivity to receptor-mediated

Table 1 Effect of maximal concentrations of insulin and cytokines on NADPH-dependent H_2O_2 generation in crude plasma membranes from 3T3 L1-cells

Crude plasma membranes were exposed to insulin and cytokines as indicated. Preincubations and assays of H_2O_2 were carried out as described in the legend to Figure 1. Values are means $\pm$ S.E.M. of five separate experiments for each condition. *Significantly different from controls ($P \leq 0.0001$); **significantly different from insulin-stimulated H_2O_2 generation ($P \leq 0.0001$) (Student's *t* test).

Additions	H_2O_2 generation (nmol/min per mg)
None	25 $\pm$ 5
Insulin (5 nM)	53 $\pm$ 10*
IGF-I (10 nM)	55 $\pm$ 11*
aFGF (0.1 nM)	42 $\pm$ 7*
bFGF (1 nM)	13 $\pm$ 4*
bFGF (1 nM) + insulin (5 nM)	30 $\pm$ 4**
PDGF AA (1 nM)	50 $\pm$ 7*
PDGF BB (1 nM)	28 $\pm$ 4
PDGF BB (10 nM) + insulin (5 nM)	35 $\pm$ 4**

Table 2 Effects of insulin and different members of the FGF and PDGF families on H_2O_2 release by intact 3T3 L1 cells

Confluent cultures of 3T3 L1-cells were incubated in PBS containing 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 10 mM glucose, 0.1% human serum albumin and 1 mM NaN_3 in the absence or presence of 10 nM insulin and/or 100 pM PDGF AA, 1 nM PDGF BB, 100 pM aFGF, 100 pM bFGF. After 10 min aliquots of the media were removed and assayed for H_2O_2 . Values are means $\pm$ S.E.M. of five separate experiments for each condition. *Significantly different from controls ($P \leq 0.001$); **significantly different from rates of insulin-stimulated H_2O_2 release ($P \leq 0.04$); ***significantly different from rates of insulin-stimulated H_2O_2 release ($P \leq 0.001$) (Student's *t* test).

Additions	H_2O_2 production (nmol/h per 10^7 cells)
None	13 $\pm$ 7
Insulin	77 $\pm$ 15*
PDGF AA	86 $\pm$ 5*
aFGF	83 $\pm$ 15*
Insulin + PDGF BB	48 $\pm$ 8**
Insulin + bFGF	32 $\pm$ 9***

stimuli after cell disruption, and Mn^{2+} was the sole cofactor required to elicit a ligand-induced modulation of NADPH-dependent H_2O_2 generation in crude plasma membranes *in vitro*. Insulin and IGF-I were active stimulators of the 3T3 L1-system (Table 1). As in mature human adipocytes [1,2], the AA and BB homodimers of PDGF, as well as the acidic and basic isoforms of FGF (FGF1 and FGF2), had antagonistic stimulatory (PDGF AA, aFGF) and inhibitory (PDGF BB, bFGF) effects on NADPH-dependent H_2O_2 generation (Table 1). bFGF inhibited basal as well as ligand-activated rates of NADPH-dependent H_2O_2 generation, whereas a consistent inhibition by PDGF BB was only achieved in the simultaneous presence of a stimulatory ligand, such as insulin (Table 1).

As in primary human adipocytes [1] and rat fat-cells [20,21], insulin also stimulated H_2O_2 release by 3T3 L1-preadipocytes resulting in a fivefold increase in H_2O_2 concentration in the media in the presence of 1 mM NaN_3 (Table 2). PDGF AA and aFGF caused increases in H_2O_2 accumulation that were comparable with that seen in the presence of insulin. By contrast, bFGF and PDGF BB consistently reduced H_2O_2 accumulation

Table 3 Improvement of the adipogenic action of insulin by heat inactivation of FCS and by lowering its concentration in the media

Cells were differentiated from confluence to day 9 without insulin (none) or with two concentrations of insulin in media supplemented with various amounts of native or heat-inactivated FCS, as indicated. Values are means $\pm$ S.E.M. of six separate experiments. * $P \leq 0.02$, ** $P \leq 0.01$ compared with control (Student's *t* test).

Additions	GPDH (m-units/mg of protein)		
	Untreated 10% FCS	Heat-inactivated 10% FCS	Heat-inactivated 5% FCS
None	50 $\pm$ 15	370 $\pm$ 150	320 $\pm$ 110
Insulin (1 μ M)	610 $\pm$ 170**	780 $\pm$ 80*	880 $\pm$ 110*
Insulin (10 nM)	490 $\pm$ 100*	1030 $\pm$ 50**	1250 $\pm$ 170**

Figure 1 Dose-response curves for stimulation of NADPH-dependent H_2O_2 generation and adipogenesis by insulin

Plasma membranes were treated with increasing concentrations of insulin and assayed for NADPH oxidase activity (Δ) as described in the Materials and methods section. Adipose conversion ($\bullet$) was assessed in terms of GPDH activity at day 9 after induction of differentiation. Values are means $\pm$ S.E.M. of three separate experiments for each condition.

in the presence of insulin. Thus the divergent effects of different PDGF and FGF isoforms in crude plasma membranes were paralleled by corresponding changes in H_2O_2 accumulation in intact cells.

Close relationship between the adipogenic effect of insulin and its stimulatory action on NADPH-dependent H_2O_2 generation

Adipose conversion is usually induced in media supplemented with 10% FCS, but can also be observed under serum-free conditions [22,23]. All serum-free culture systems that have successfully been used for adipocyte differentiation contain iron, which can catalyse the non-enzymic generation of reactive oxygen species. Therefore we chose a medium supplemented with FCS. However, FCS is not only a source of hormones and growth factors, but also contains other compounds that are relevant in the context of the present experiments, e.g. catalase and peroxidases. To overcome the difficulties arising from the complex composition of FCS, the serum was heat-inactivated and its concentration was lowered to 5%. In the presence of 10% native FCS, differentiation was optimal at 1 μ M insulin, as has also been observed by others (Table 3). Interestingly, heat inactivation of FCS reduced the concentration of insulin required for adipose conversion by at least two orders of magnitude, i.e. from 1 μ M

Figure 2 Correlation between the antagonistic effects of different FGF and PDGF isoforms on NADPH-dependent H_2O_2 generation in plasma membranes and their adipogenic and antiadipogenic actions in intact cells

(a,c) Antagonistic effects of different FGF and PDGF isoforms on NADPH-dependent H_2O_2 generation. (a) Effects of aFGF (□) and bFGF (■) on basal rates of NADPH-dependent H_2O_2 generation. A consistent inhibition of NADPH-dependent H_2O_2 generation by PDGF BB (▲) was only observed in the presence of a stimulatory ligand. Therefore the inhibitory effects of PDGF BB were assessed in the presence of 5 nM insulin (c). △, Stimulation of basal rates of NADPH-dependent H_2O_2 generation by PDGF AA. (b,d) Effects of different FGF and PDGF isoforms on expression of the adipocyte phenotype induced by insulin, dexamethasone and IBMX. Differentiation was induced by 0.5 mM IBMX, 0.5 μ M dexamethasone and 10 nM insulin either alone (●) or in combination with various concentrations of aFGF (□) and bFGF (■) (b), or PDGF AA (△) and PDGF BB (▲) (d). In contrast with dexamethasone and IBMX which were present for the first 2 days only, insulin and cytokines were present during the entire course of cell cultures. Values are means $\pm$ S.E.M. of five separate experiments for each condition.

to 10 nM (Table 3), indicating that native FCS contains heat-labile inhibitors of differentiation. Reducing FCS concentrations from 10 to 5% resulted in a further improvement in insulin sensitivity (Table 3 and Figure 1). The adipogenic action of insulin was maximal at 1–10 nM under these conditions, and then declined (Figure 1). The latter phenomenon has also been observed by others using chemically defined media [22,23].

The dose-response curves for stimulation of NADPH-dependent H_2O_2 generation by insulin in crude plasma membranes from 3T3 L1-cells were virtually identical with those obtained in human fat-cell ghosts. Half-maximal effects were observed at approx. 3 pM. The adipogenic action of the hormone was apparent at similar concentrations with half-maximal stimulation occurring at approx. 30 pM insulin. These results suggest that a receptor-mediated change in the intracellular redox state may be required for the adipogenic action of insulin.

Adipogenic and antiadipogenic effects of FGF and PDGF isoforms

As insulin stimulated NADPH-dependent H_2O_2 generation with a dose-response relation similar to its adipogenic effects, we next examined whether any parallels existed between the antagonistic effects of different PDGF and FGF isoforms on NADPH-dependent H_2O_2 generation and their influences on adipocyte differentiation (Figure 2).

Indeed, the effects of different members of both growth factor families on adipocyte differentiation closely corresponded to

their regulatory influences on NADPH-dependent H_2O_2 generation in plasma membranes. The stimulatory effect of aFGF on NADPH-dependent H_2O_2 generation was half-maximal at approx. 5 pM, reached peak levels between 20 pM and 0.2 nM, and then declined (Figure 2a). Accordingly, this FGF isoform accelerated the expression of the adipocyte phenotype induced by insulin, dexamethasone and IBMX only at low concentrations; the stimulatory effect peaked at 10 pM aFGF and resulted in a maximal increase in GPDH activity of 40% above control levels. At higher concentrations, the stimulatory effect of aFGF declined with net inhibition occurring at concentrations above 1 nM (Figure 2b). By contrast, bFGF suppressed both NADPH-dependent H_2O_2 generation and adipocyte differentiation over the whole range of concentrations tested, and its effect was half-maximal at approx. 10 pM in both conditions.

The antagonistic effects of PDGF AA and PDGF BB on NADPH-dependent H_2O_2 generation were also paralleled by corresponding effects on adipocyte differentiation induced by insulin, dexamethasone and IBMX (Figures 2c and 2d). Half-maximal increases in NADPH-dependent H_2O_2 generation were observed at approx. 3 pM PDGF AA, and half-maximal inhibition of insulin-stimulated rates of H_2O_2 production occurred at approx. 10 pM PDGF BB. These values correspond well with the concentrations required to elicit comparable effects on fat-cell differentiation, which occurred at approx. 30 pM for each PDGF isoform.

Figure 3 Insulinomimetic effects of aFGF and PDGF AA on adipocyte differentiation: antagonistic actions of bFGF and PDGF BB

Confluent cells were treated with dexamethasone and IBMX either alone (○) or in combination with 10 nM insulin (●), 0.5 nM PDGF AA (△), 0.1 nM aFGF (□), 0.5 nM PDGF BB (▲) or 0.1 nM bFGF (■). After 2 days, dexamethasone and IBMX were omitted, and cultures were continued in the presence of insulin or growth factors alone for various intervals. Values are means $\pm$ S.E.M. of three separate experiments.

PDGF AA and aFGF not only accelerated the expression of the adipocyte phenotype induced by insulin, dexamethasone and IBMX, but were also adipogenic in themselves. This is illustrated in Figure 3, in which the effects of different members of the PDGF and FGF families on adipose conversion (in terms of GPDH activity) are compared with those of insulin. When applied at concentrations of 0.5 nM and 0.1 nM respectively, PDGF AA and aFGF were nearly as effective at promoting the expression of the adipocyte phenotype as a maximal concentration of insulin. In contrast, bFGF and PDGF BB not only inhibited the adipogenic effects of insulin, but also suppressed the small increase in GPDH activity seen in the presence of dexamethasone and IBMX alone.

Augmentation of adipocyte differentiation by exogenously supplied reactive oxygen species and inhibition by antioxidants

As the effects of different PDGF and FGF isoforms on adipocyte differentiation closely corresponded to their actions on NADPH-dependent H_2O_2 generation, the effects of exogenously supplied H_2O_2 on adipose conversion induced by insulin, dexamethasone and IBMX were explored next. The oxidation of hypoxanthine by xanthine oxidase was used for the continuous production of reactive oxygen species, this results in the generation of superoxide radicals and H_2O_2 .

Figure 4 shows the effects of various concentrations of xanthine oxidase in the presence of 1 mM hypoxanthine on GPDH activity at day 9 after the induction of differentiation by insulin, dexamethasone and IBMX. Adipose conversion was accelerated in the presence of hypoxanthine/xanthine oxidase, and this effect was preceded by a lag phase of 4–6 days. The stimulatory effect was maximal with the addition of 1 μ -unit/ml xanthine oxidase; GPDH activity and triacylglycerol content were increased 30 and 50% respectively above control levels (Figure 4). The stimulatory effect of hypoxanthine/xanthine oxidase was abolished in the simultaneous presence of 30 units/ml catalase, whereas superoxide dismutase (15 units/ml) caused a further increase in adipose conversion (Table 4). These results suggest that H_2O_2 rather than the superoxide anion was the major reactive oxygen

Figure 4 Acceleration of adipocyte differentiation by exogenously supplied hypoxanthine/xanthine oxidase

(a) Confluent cells were differentiated in the absence (dexamethasone, IBMX) (○) or presence of 10 nM insulin (dexamethasone, IBMX, insulin) either alone (●) or in combination with 1 μ -unit of xanthine oxidase and 1 mM hypoxanthine (△). Values are means $\pm$ S.E.M. of duplicate determinations. (b) Confluent cells were differentiated as described above. The media contained 1 mM hypoxanthine and various concentrations of xanthine oxidase, as indicated. Cells were harvested on day 9. □, GPDH; ■, triacylglycerol. Values are means $\pm$ S.E.M. of three separate experiments, each performed in duplicate. * $P \leq 0.05$, ** $P \leq 0.01$ compared with controls (no xanthine oxidase added) (Student's *t* test).

Table 4 Specificity of the adipogenic action of exogenously supplied xanthine oxidase/hypoxanthine

Cells were differentiated from confluence to day 9 in the absence or presence of xanthine oxidase/hypoxanthine, superoxide dismutase and catalase, as indicated. Values are means $\pm$ S.E.M. of three separate experiments. *Significantly different from controls ($P \leq 0.001$) (Student's *t* test).

Additions	GPDH (m-units/ mg of protein)
None	930 $\pm$ 50
Xanthine oxidase/hypoxanthine (1 μ -unit/ml, 1 mM)	1220 $\pm$ 150*
Xanthine oxidase/hypoxanthine + superoxide dismutase (15 units/ml)	1410 $\pm$ 170*
Xanthine oxidase/hypoxanthine + catalase (30 units/ml)	990 $\pm$ 140

intermediate responsible for the enhancement of differentiation. Accordingly, exogenously supplied H_2O_2 produced the same qualitative pattern of effects. However, the rates of H_2O_2 degradation showed considerable variability between cultures, and this complicated the choice of appropriate H_2O_2 concentrations (results not shown).

Figure 5 Antiadipogenic action of *N*-acetylcysteine

Confluent cells were differentiated as described in the Materials and methods section (insulin, dexamethasone, IBMX) in the absence (○) or presence of 1.25 mM (□) or 2.5 mM (▲) *N*-acetylcysteine or cultured in DMEM supplemented with 5% FCS (●). (a) Cells were harvested as indicated and assayed for GPDH activity. (b) Triacylglycerol content of the cells was determined at day 11 after confluence. Values are means $\pm$ S.E.M. of three experiments, each performed in duplicate. * $P \leq 0.001$, ** $P \leq 0.0001$ compared with control (Student's *t* test).

Figure 6 Inhibition of adipocyte differentiation by ascorbic acid

Confluent cells were differentiated as described in the Materials and methods section in the absence (●) or presence (▲) of 100 μ M ascorbic acid. Values are means $\pm$ S.E.M. of three separate experiments. * $P \leq 0.05$ compared with the value obtained in the absence of ascorbic acid (Student's *t* test).

Figure 5 shows the effects of various concentrations of *N*-acetylcysteine, which serves as a scavenger for reactive oxygen intermediates and as a precursor for glutathione, on expression of GPDH activity. It suppressed the differentiation of 3T3 L1-

Table 5 Specificity of the antiadipogenic action of ascorbate

Cells were differentiated as described in the Materials and methods section from confluence to day 11 in the absence or presence of 100 μ M ascorbic acid or 100 μ M dehydroascorbic acid. Values are means $\pm$ S.E.M. of three separate experiments.

Additions	GPDH (m-units/mg of protein)
None	1360 $\pm$ 130
Ascorbic acid	820 $\pm$ 110
Dehydroascorbic acid	1660 $\pm$ 150

Table 6 Reversal of the antiadipogenic effect of bFGF by exogenous hypoxanthine/xanthine oxidase

Confluent cells were differentiated as described in the Materials and methods section in the absence or presence of 50 pM bFGF, either alone or in combination with 1 mM hypoxanthine and 1 μ -unit/ml xanthine oxidase. Cells were harvested at day 11. Values are means $\pm$ S.E.M. of five separate experiments, each performed in duplicate. *The inhibitory action of bFGF on insulin-stimulated rates of GPDH activation was partially reversed by xanthine oxidase/hypoxanthine ($P \leq 0.01$; Student's *t* test).

Additions		GPDH activation (m-units/mg of protein)
Activator	Inhibitor	
Insulin	None	1160 $\pm$ 245
Insulin, xanthine oxidase/hypoxanthine	None	1420 $\pm$ 320
Insulin	bFGF	390 $\pm$ 262
Insulin, xanthine oxidase/hypoxanthine	bFGF	945 $\pm$ 286*

preadipocytes, as expected. The antiadipogenic effect of *N*-acetylcysteine was concentration-dependent. In the presence of 2.5 mM *N*-acetylcysteine, GPDH activity was approx. 25% of control levels. The decrease in GPDH activity was accompanied by a corresponding reduction in cellular triacylglycerol content (Figure 5b). Cell number was not influenced over the whole range of *N*-acetylcysteine concentrations tested.

In contrast with *N*-acetylcysteine, ascorbate caused an increase in cell proliferation that was associated with a moderate increase in GPDH activity (Figure 6). The stimulatory effect was followed by an abrupt cessation of GPDH expression around day 6 resulting in a marked inhibition of adipose conversion. The latter effect was not shared by dehydroascorbate (which acts as a pro-oxidant), indicating that the antiadipogenic action of ascorbate was due to its antioxidant properties (Table 5). Even though the mechanisms responsible for the initial stimulation of cell proliferation and GPDH activity seen in the presence of 100 μ M ascorbic acid remain to be clarified, the results consistently show that adipose conversion is augmented by exogenously supplied H_2O_2 and is specifically inhibited by antioxidants that are unrelated in structural terms.

Reversal of the antiadipogenic effect of bFGF in the presence of hypoxanthine/xanthine oxidase

As the effects of aFGF and bFGF on adipocyte differentiation closely corresponded to their effects on NADPH-dependent H_2O_2 generation, we next examined whether the FGF-induced inhibition of adipose conversion could be overcome by hypoxanthine/xanthine oxidase (Table 6). Addition of 50 pM

bFGF to confluent cultures of 3T3 L1-preadipocytes supplemented with insulin, dexamethasone and IBMX blocked differentiation, and the cells retained the typical fibroblast morphology. In contrast, in the simultaneous presence of hypoxanthine/xanthine oxidase, the antiadipogenic effect of bFGF was markedly attenuated or even reversed, depending on the bFGF concentration used. To show that bFGF remained functional we also used bFGF that had been pretreated with hypoxanthine/xanthine oxidase for 3.5 h at 37 °C. The preparation retained its antiadipogenic effect, indicating that it was biologically active.

DISCUSSION

In the preceding paper [24] it was shown that human fat-cells possess a plasma-membrane-bound H_2O_2 -generating system that meets all the criteria of a universal signal-transducing system for hormones and cytokines, and may link ligand binding to cell-surface receptors to changes in the intracellular redox equilibrium. In this report it is demonstrated that this type of redox activity is also present in adipogenic 3T3 L1-preadipocytes, where it may be important for adipose conversion and maintenance of the differentiated state.

The latter hypothesis is based on several lines of evidence. First, it has been a common experience that the adipogenic effect of insulin is only apparent at pharmacological concentrations in the presence of native calf serum [9]. It has therefore been proposed that the adipogenic action of insulin may be mediated via the receptor for IGF-I, which binds insulin with an affinity approx. 70 times lower than its affinity for IGF-I [25,26]. The present finding shows that heat treatment of FCS results in a marked improvement in insulin sensitivity which is probably due to the inactivation of heat-labile inhibitors of adipocyte differentiation. Under the conditions used, the adipogenic action of insulin was half-maximal at approx. 10 pM, a concentration at which insulin does not interact with the IGF-I receptor [26]. Interestingly, insulin stimulated NADPH-dependent H_2O_2 generation at concentrations similar to those at which it induced the expression of the adipocyte phenotype under the conditions used. This observation supported the idea that activity of the stimulus-sensitive H_2O_2 -generating system contributes to, or is required for, expression of the adipocyte phenotype.

Second, even though multiple cell-surface receptors exist for FGF and PDGF that display (partial) isoform specificity and may mediate distinct signals [12–16,27–31], the biological effects of individual members of the FGF and PDGF families are remarkably similar in most target cells [10,13]. Examples of divergent responses to the three PDGF isoforms are rare [30,31], and very few cases have been reported where aFGF was able to induce a cellular effect that could not be detected with bFGF [29]. It was therefore surprising that aFGF and bFGF as well as PDGF AA and PDGF BB had antagonistic effects on NADPH-dependent H_2O_2 generation in crude plasma membranes and intact cells. These unexpected results prompted a search for isoform-specific cellular responses. Indeed, aFGF and PDGF AA (which stimulated H_2O_2 generation) accelerated adipose conversion induced by insulin in the presence of dexamethasone and IBMX, and were adipogenic in themselves. In contrast, bFGF and PDGF BB, which suppressed H_2O_2 generation, prevented the expression of the adipocyte phenotype triggered by insulin, dexamethasone and IBMX. In addition, the concentrations of FGFs and PDGFs required to modulate NADPH-dependent H_2O_2 generation in crude plasma membranes were nearly identical with those eliciting adipogenic or antiadipogenic effects in intact cells. The observation that different members of the FGF

and PDGF growth factor families have divergent effects on adipose conversion is novel and may explain the conflicting results of earlier studies, which did not discriminate between isoforms, about the effects of FGF and PDGF on expression of the adipocyte phenotype [5,8,23,32].

The investigations into the cellular responses to different members of the PDGF and FGF families were undertaken because of their divergent influences on NADPH-dependent H_2O_2 generation. Therefore the striking parallels between the effects of different PDGF and FGF isoforms on H_2O_2 generation in crude plasma membranes and their effects on expression of the adipocyte phenotype alone provide convincing evidence that receptor-mediated changes in the intracellular redox equilibrium are in fact important to the regulation of fat-cell differentiation.

Third, the inhibitory effects of bFGF and PDGF BB were mimicked by *N*-acetylcysteine and ascorbic acid, two well-studied antioxidants that are unrelated in structural terms [33–35]. In contrast with *N*-acetylcysteine, ascorbate stimulated cell proliferation and this may, in part, explain why GPDH levels were slightly increased during the first days of differentiation. However, the main effect of ascorbate was clearly an inhibitory one, which was specific because dehydroascorbate had no antiadipogenic effects. Conversely, hypoxanthine/xanthine oxidase, when added as a source for continuous generation of reactive oxygen species, not only accelerated adipose conversion induced by insulin, dexamethasone and IBMX, but also caused a reversal of the inhibitory action of bFGF, as might be expected if a functional link exists between NADPH-dependent H_2O_2 generation and cell differentiation. However, unlike aFGF and PDGF BB, exogenously supplied reactive oxygen species could not substitute for insulin as an inducing agent under various conditions tested. We cannot exclude that the concentrations of H_2O_2 achieved in the presence of hypoxanthine/xanthine oxidase were suboptimal. It is also possible that exogenous H_2O_2 has limited access to one or more intracellular sites that are relevant for adipose conversion.

However, it is well established that insulin and other growth factors generate multiple and independent signals originating from the occupied receptors [36,37]. Therefore it remains likely that a ligand-induced redox change, although necessary, may not be the sole pathway by which insulin, aFGF and PDGF AA induce the expression of the adipocyte phenotype. The concept that signalling by insulin and other growth factors takes place via multiple signal-transduction systems may also explain why TNF- α , a stimulator of NADPH-dependent H_2O_2 generation, had antiadipogenic effects (results not shown).

H_2O_2 and oxygen radicals have long been implicated as second messengers in regulating cell division, chemotaxis and the action of insulin and other hormones [3]. More recent precedents for reactive oxygen intermediates as second messengers arise from previous work showing that these compounds modulate DNA binding of certain transcription factors, such as nuclear factor κB , Jun/AP1 or helix-loop-helix members [33,34,38–42]. These nuclear changes are believed to confer longer-term changes in gene transcription associated with both mitogenic and differentiating signals, consistent with the current findings and previous work of others demonstrating that reactive oxygen species may be involved in mediating the proliferative effects of hormones and cytokines in fibroblasts and osteoclasts [43,44].

The diet contains numerous compounds of oxidant and antioxidant nature, and considerable efforts are currently being made to elucidate the possible protective effects of antioxidant vitamins and β -carotene [45]. Regulation of gene expression by specific nutrients is another major frontier for nutrition scientists [46]. Together with most recent findings on redox regulation of

non-receptor tyrosine kinases [47–49] and of a variety of transcription factors and translational activators [33,34,38–42,50,51], our findings raise the possibility that interaction of oxygen-derived messengers with antioxidant nutrients may represent an important mechanism of nutrient gene interaction that has received relatively little attention until now.

We are indebted to B. Sattel and B. Gail for expert technical assistance. This work was supported by a grant from the Bundesministerium für Forschung und Technologie, Bonn, Germany.

REFERENCES

- Krieger-Brauer, H. I. and Kather, H. (1992) *J. Clin. Invest.* **89**, 1006–1013
- Mukherjee, S. P. (1980) *Biochem. Pharmacol.* **29**, 1239–1246
- Ramasarma, T. (1982) *Biochim. Biophys. Acta* **694**, 69–93
- Green, H. and Meuth, M. (1974) *Cell* **3**, 127–133
- Sadowsky, H. B., Wheeler, T. T. and Young, D. A. (1992) *J. Biol. Chem.* **267**, 4722–4731
- Hayashi, J., Nixon, T., Morikawa, M. and Green, H. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3969–3972
- Torti, F. M., Torti, S. V., Larrick, J. W. and Ringold, G. M. (1989) *J. Cell Biol.* **108**, 1105–1113
- Navre, M. and Ringold, G. M. (1989) *J. Cell Biol.* **109**, 1857–1863
- Sul, H. S. (1989) *Curr. Opin. Cell Biol.* **1**, 1116–1121
- Heldin, C. H. (1992) *EMBO J.* **11**, 4251–4259
- Miyamoto, M., Naruo, K. I., Seko, C., Matsumoto, S., Kondo, T. and Kurokawa, T. (1993) *Mol. Cell. Biol.* **13**, 4251–4259
- Givol, D. and Yayon, A. (1992) *FASEB J.* **6**, 3362–3369
- Ledoux, D., Gannoun-Zaki, L. and Barritault, D. (1993) *Prog. Growth Factor Res.* **4**, 107–120
- Imamura, T., Engleka, K., Zhan, X. et al. (1990) *Science* **249**, 1567–1570
- Nakanishi, T., Kihara, K., Mizuno, K., Masamune, Y., Yoshitake, Y. and Nishikawa, K. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5216–5220
- Burrus, L. W., Zuber, M. E., Lueddecke, B. A. and Olwin, B. B. (1992) *Mol. Cell. Biol.* **12**, 5600–5609
- Kather, H. and Wieland, E. (1984) *Methods Enzym. Anal.* (3rd Ed.) **6**, 510–518
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. and James, D. E. (1992) *J. Cell Biol.* **117**, 1181–1196
- Mukherjee, S. P. and Lynn, W. S. (1977) *Arch. Biochem. Biophys.* **184**, 69–76
- May, J. M. and de Haen, C. (1979) *J. Biol. Chem.* **254**, 2214–2220
- Hauner, H. (1990) *Exp. Clin. Endocrinol.* **127**, 865–872
- Schmidt, W., Pöhl-Jordan, G. and Löffler, G. (1990) *J. Biol. Chem.* **265**, 15489–15495
- Krieger-Brauer, H. I. and Kather, H. (1995) *Biochem. J.* **307**, 543–548
- Smith, P., Wise, L. S., Berkowitz, R., Wan, C. and Rubin, S. (1988) *J. Biol. Chem.* **263**, 9402–9408
- Humbel, R. E. (1990) *Eur. J. Biochem.* **190**, 445–462
- Yayon, A., Zimmer, Y., Guo-Hong, S., Avivi, A., Yarden, Y. and Givol, D. (1992) *EMBO J.* **11**, 1885–1890
- Ron, D., Reich, R., Chedid, M. et al. (1993) *J. Biol. Chem.* **268**, 5388–5394
- Parker, T. G., Chow, K. L., Schwartz, R. J. and Schneider, M. D. (1992) *J. Biol. Chem.* **267**, 3343–3350
- Kondo, T., Konishi, F., Inui, H. and Inagami, T. (1993) *J. Biol. Chem.* **268**, 4458–4464
- Koyama, N., Morisaki, N., Saito, Y. and Yashida, S. (1992) *J. Biol. Chem.* **267**, 22806–22812
- Broad, T. E. and Ham, R. G. (1983) *Eur. J. Biochem.* **135**, 33–39
- Staal, F. J. T., Roederer, M., Herzenberg, L. A. and Herzenberg, L. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9943–9947
- Toledano, M. B. and Leonard, W. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4328–4332
- Rose, R. G. and Bode, A. M. (1993) *FASEB J.* **7**, 1135–1142
- Wilden, P. A., Backer, J. M., Kahn, C. R., Catill, D. A., Schroeder, G. J. and White, M. F. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3358–3362
- Mohammadi, M., Dionne, C. A., Li, W. et al. (1992) *Nature (London)* **358**, 681–684
- Hayashi, T., Ueno, Y. and Okamoto, T. (1993) *J. Biol. Chem.* **268**, 11380–11388
- Abate, C., Patel, L., Rauscher III, F. J. and Curran, T. (1990) *Science* **249**, 1157–1161
- Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E. and Curran, T. (1992) *EMBO J.* **11**, 3323–3335
- Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) *EMBO J.* **12**, 2005–2015
- Pognonec, P., Kato, H. and Roeder, R. G. (1992) *J. Biol. Chem.* **267**, 24563–24567
- Murrell, G. A. C., Francis, M. J. O. and Bromley, L. (1990) *Biochem. J.* **265**, 659–665
- Garrett, I. R., Boyce, B. F., Oreffo, R. O. C., Bonewald, L., Poser, J. and Mundy, G. R. (1990) *J. Clin. Invest.* **85**, 632–639
- Slater, T. F. and Block, G. (eds.) (1991) *Am. J. Clin. Nutr.* **53**, 189S–396S
- Clark, S. P. and Abraham, S. (1992) *FASEB J.* **6**, 3146–3152
- Bauskin, A. R., Alkalay, J. and Ben-Neriah, Y. (1991) *Cell* **66**, 685–696
- Schieven, G. L., Kirihara, J. M., Burg, D. L., Geahlen, R. L. and Ledbetter, J. (1993) *J. Biol. Chem.* **268**, 16688–16692
- Veillette, A., Dumont, S. and Fournel, M. (1993) *J. Biol. Chem.* **268**, 17547–17553
- Klausner, R. D., Rouault, T. A. and Harford, J. B. (1993) *Cell* **72**, 19–28
- Malter, J. S. and Hong, Y. (1991) *J. Biol. Chem.* **266**, 3167–3171