Interaction of glucose and long chain fatty acids (C18) on antioxidant defences and free radical damage in porcine vascular smooth muscle cells in vitro

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Abstract

Aims/hypothesis. Abnormalities of glucose and fatty acid metabolism in diabetes are believed to contribute to the development of oxidative stress and the long term vascular complications of the disease; therefore the interactions of glucose and long chain fatty acids on free radical damage and endogenous antioxidant defences were investigated in vascular smooth muscle cells.

Methods. Porcine vascular smooth muscle cells were cultured in 5 mmol/l or 25 mmol/l glucose for 10 days. Fatty acids, stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and α -linolenic acid (18:3) were added with defatted bovine serum albumin as a carrier for the final three days.

Results. Glucose (25 mmol/l) alone caused oxidative stress in the cells as evidenced by free radical-mediated damage to DNA, lipids, and proteins. The addition of fatty acids (0.2 mmol/l) altered the profile of free

radical damage; the response was J-shaped with respect to the degree of unsaturation of each acid, and oleic acid was associated with least damage. At a lower concentration α -linolenic acid (0.01 mmol/l) was markedly different in that, when added to 25 mmol/l glucose it resulted in a decrease in free radical damage to DNA, lipids and proteins. This was accompanied by a marked increase in antioxidant and glutathione concentrations as well as by increased gene expression of γ -glutamylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis.

Conclusions/Interpretation. The results clearly show that glucose and fatty acids interact in the production of oxidative stress in vascular smooth muscle cells.

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Keywords Glucose, stearic acid, oleic acid, linoleic acid, α -linolenic acid, vascular smooth muscle cells, oxidative stress.

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Abbreviations: VSMC vascular smooth muscle cells; DMEM Dulbecco's modified Eagle medium; NG normal glucose; HG high glucose; GSH glutathione; GSSG glutathione disulphide; MDA malondialdehyde; BHT butylated hydroxytoluene; PBST phosphate buffered saline with 0.2% Tween; DNPH di-nitrophenylhydrazine; GAPDH glycerol-3-phosphate dehydrogenase; γ -GCS γ -glutamylcysteine synthetase; SOD superoxide dismutase; SSC standard sodium citrate.

Increased glucose availability leads to many changes in cellular metabolism resulting from glucose autooxidation, glycation, de novo synthesis of diacylglycerol, and increased free radical production [1, 2].

Diabetes is a disease characterised not only by abnormalities of glucose metabolism but also of fatty acid metabolism. Polyunsaturated fatty acids, recommended in preference to saturated fatty acids, as a dietary means of cholesterol reduction [3, 4], are highly susceptible to free radical attack. Dietary increases of polyunsaturated fatty acids within a nutritionally relevant range lead to enhanced oxidative damage of DNA [5] and proteins [6], effects that could be caused indirectly by lipid peroxidation [5, 7]. Simplistically, one might expect the degree of free radical-induced

damage to be linearly related to the number of unsaturated bonds in each fatty acid and to the pre-existing antioxidant status of the cells. However, there are a number of pathways by which fatty acids can affect cellular metabolism that might alter this simple relationship [8, 9]. Furthermore, differences between the acute and longer-term effects of fatty acids on cellular function will depend on their subsequent metabolism within the cell and that tissue's profile of elongase and desaturase enzymes.

Since free radical-induced damage of cellular structures is thought to play an important role in atherosclerosis, and the late complications of diabetes [10], the purpose of this project was to investigate the interactions of glucose and long chain fatty acids of the C18 lineage in aortic VSMC. In view of the importance of GSH and antioxidant enzymes in hyperglycaemia-induced oxidative stress we have investigated the interactions of glucose and fatty acids on GSH synthesis and regulation of antioxidant defences in porcine VSMC and on free radical-mediated damage to lipids, proteins, and DNA.

Materials and methods

D-Glucose was purchased from BDH (Poole, England, UK). All tissue culture reagents were obtained from Invitrogen (Paisley, Scotland, UK) and all other chemicals were obtained from Sigma (Poole, England, UK) unless otherwise stated.

Cell culture. Porcine aortae were obtained from the local abattoir and VSMC isolated for culture in DMEM with 10% (v/v) FCS as described [11]. Cells were confirmed as VSMC by positive immunofluorescence staining with a monoclonal antibody against smooth muscle α -actin. All experiments were carried out using cells of passage 2 to 5.

Cells were cultured in either 5 mmol/l (NG) or 25 mmol/l (HG) glucose for 10 days. For the final 3 days of this experimental period 0.2 mmol/l fatty acids and defatted bovine serum albumin (BSA) were added in a ratio of 5 to 6:1 [8, 12]. Fatty acids used were the saturate, stearic acid (18:0), the monounsaturate, oleic acid (18:1), and the polyunsaturates, linoleic (18:2) and α -linolenic acids (18:3). Prior to use fatty acids were tested for purity by GC analysis; purity was greater than 97% in all cases. The effects of all fatty acids were tested at a concentration of 0.2 mmol/l, which is within the physiological range for plasma stearic and oleic acids but is supraphysiological for linoleic and α -linolenic acids. Therefore, more physiological concentrations of linoleic acid (0.1 mmol/l) and α -linolenic acid (0.01 mmol/l) were also used, with adjustment of BSA concentration.

Fatty acid profile. VSMC were harvested into cold HBSS containing 10 mg% butylated hydroxytoluene (BHT). Cell extracts (500 μ l) were mixed with chloroform/methanol (2:1, 4 ml) containing BHT and allowed to extract for 30 min. Calcium chloride (2 ml, 14 mmol/l) was added, mixed and centrifuged (200 g for 10 min). The samples were then methylated [13] and the lower lipid layer of the extract evaporated to dryness under nitrogen and re-dissolved in 2 ml boron trifluoride-methanol. The solution was then incubated at 60°C for 30 min and then allowed to cool. Petroleum spirit (2 ml) and distilled water (2 ml) were added, mixed and allowed to stand for 20 min until

the layers separated. The top petroleum layer was transferred into a clean pre-weighed glass vial and evaporated to dryness under nitrogen. Fatty acid was re-dissolved in 250 μ l petroleum spirit containing 10 mg% BHT. The total fatty acid composition was analysed by manual injection of 2 μ l aliquots into a Hewlett Packard (HP) 6890 gas chromatograph system equipped with an HP chemstation and a 25 m $\times$ 0.22 mm I.D. BP 20, fused silica column, film thickness 0.25 μ m (Scientific Glass Engineering, Milton Keynes, UK). Helium gas flow rate was 1.5 to 2.0 ml per min, flame ionisation detector temperature 300°C and injector temperature 240°C. The oven temperature was programmed from 70 to 160°C at 25°C per min then 160 to 250°C at 3°C per min, and isothermal for 20 min. The fatty acid methyl esters were identified according to their retention times compared with known standards.

Oxidative stress. At the end of the experimental periods, VSMC were harvested for measurement of malondialdehyde (MDA), glutathione (GSH), antioxidant enzyme activities (total SOD, GPX) and protein activities as previously described [11]. The enzyme activities of glutathione reductase and catalase were measured in the cell extracts using methods adapted for automation on the Cobas Fara centrifugal analyser (Roche Products, Welwyn Garden City, Herts., UK) [14, 15, 16].

DNA fragmentation. The alkaline single cell gel electrophoresis or comet assay is a microgel electrophoretic technique for the detection of single strand DNA breaks in individual cells [18]. The assay was carried out as previously described [19]. Briefly, VSMC were trypsinised and the number of cells adjusted to a concentration of 1×10^4 cells/ml. DNA damage was quantified using the image analysis system Komet 3.1 (Kinetic Imaging, Liverpool, UK). The comet appears as a head that contains undamaged DNA, and a tail that represents the damaged DNA. The amount of damage is indicated by both the intensity of the stain and the length of the comet tail.

Protein carbonyl. Protein carbonyls were measured according to an ELISA method [6]. The protein from the sample is non-specifically adsorbed to a 96 well microplate following reaction with 2,4-dinitrophenylhydrazine (DNPH), and probed with a biotinylated anti-DNPH antibody (anti-dinitrophenylhydrazine-KLH, rabbit IgG (H+L) fraction, biotin-XX conjugate, Molecular Probes, Eugene, Ore., USA), followed by streptavidin-linked horseradish peroxidase (Amersham Life Science, Little Chalfont, Bucks., UK), and the substrate [H_2O_2 (0.4 μ l/ml), ortho-phenylenediamine (0.6 mg/ml) in citric acid (1.05 mmol/l) in phosphate buffer (pH 5.6)]. The samples were incubated for 15 to 20 min and the reaction stopped by the addition of sulphuric acid (2.5 mol/l) prior to absorbance measurements ($\lambda=490\text{--}405$ nm).

Design of primers. Primers were designed using published human glycerol 3-phosphate dehydrogenase (GAPDH) [19], γ -glutamylcysteine synthetase (γ -GCS) heavy subunit (γ -GCS_H) [20], γ -GCS light subunit (γ -GCS_L) [21] and human catalase cDNA sequences; for primer sequences see Table 1.

Extraction of total RNA and Semi-Quantitative PCR. Total RNA was extracted according to the acid-guanidinium thiocyanate-phenol-chloroform method [22]. Reverse transcription and subsequent PCR was carried out as previously detailed [23]. Briefly, RNA (1 μ g) was reverse transcribed using an oligo (dt) primer and Superscript Rnase H⁻ reverse transcriptase system (Life Technologies). Equal amounts (2 μ l) of the reverse transcription reactions were subjected to PCR amplification at a final concentration of $1 \times$ PCR buffer (50 mmol/l KCL,

Table 1. Primer sequences of GAPDH, catalase and γ -GCS subunits

	Sense	Antisense
γ -GCS _H Acc No M90656	TTGGAGGCCATGTGGACC	AACTCCCTCATCCATCTGG
γ -GCS _L Acc No L35546	AACCAAGTTAATCTTGCCTCC	TGCCTCAATGACACCATTTAC
GAPDH Acc No U48832	GGAATTCAGGGACTCATGACCACGG	GGAATTCCTTGGAGGCCATGTGGACC
Catalase Acc No X04076	GGAATTCCTTCGACCCAAGCAACATGC	GGAATTCCTTGTCCAGAAGAGCCTGG

Table 2. Effect of the addition of fatty acids on fatty acid profile of VSMC as measured by GC analysis. Cells were grown in 5 mmol/l (NG) or 25 mmol/l (HG) glucose for 10 days with

0.03 mmol/l (B) defatted BSA and 0.2 mmol/l of either stearic, oleic, linoleic, or α -linolenic acids added for the final 3 days of this experimental period

Fatty Acid		stearic				oleic				linoleic				α -linolenic			
		NG	HG	NGB	HGB	NGB	HGB	NGB	HGB	NGB	HGB	NGB	HGB	NB	HB	NB	HB
myristic	14.0	1.17	1.30	1.13	1.10	0.93	1.07	1.13	1.13	1.73	1.83	3.27	2.73				
palmitic	16.0	19.93	18.83	19.73	18.70	14.10	14.70	13.80	13.93	8.87	19.67	18.87	19.07				
palmitoleic	16.1 ω 7	4.47	5.00	4.67	4.83	4.87	5.43	2.27	2.40	2.87	3.00	6.50	7.07				
stearic	18.0	21.40	20.87	20.87	20.97	17.03	15.90	6.07	7.40	7.60	7.83	15.40	16.20				
oleic	18.1 ω 9	32.20	33.77	28.87	29.13	25.97	26.87	57.13	54.00	10.60	11.40	20.10	21.77				
linoleic	18.2 ω 6	1.30	1.20	1.40	1.30	1.53	1.43	0.77	0.80	32.13	29.33	1.63	1.60				
γ -linolenic	18.3 ω 6	—	—	—	—	—	—	—	—	4.63	4.83	—	—				
α -linolenic	18.3 ω 3	—	—	—	—	—	—	—	—	—	—	4.23	2.03				
morotic	18.4 ω 3	—	—	—	—	—	—	—	—	—	—	1.27	0.33				
d-h-g-linolenic	20.3 ω 6	1.13	1.10	1.23	1.30	1.63	1.50	0.93	0.83	3.63	3.13	1.17	1.33				
arachidonic	20.4 ω 6	8.40	7.83	11.17	11.33	18.63	17.90	7.70	8.83	10.80	12.13	8.07	8.47				
eicosapentanoic	20.5 ω 3	0.90	1.10	0.77	0.80	1.83	1.90	0.57	0.60	TRACE	TRACE	10.13	8.27				
docosatetranoic	22.4 ω 6	1.47	1.30	1.90	1.70	1.33	1.20	1.97	1.83	2.20	2.37	0.67	0.70				
docosapentanoic	22.5 ω 3	4.53	4.27	5.30	5.33	7.53	7.17	4.93	4.97	3.37	2.67	6.57	7.70				
docosahexanoic	22.6 ω 3	3.07	3.47	2.97	3.50	4.67	4.90	2.73	3.13	1.47	1.80	2.13	2.80				

Individual results are expressed as percentage of total fatty acid content and as the mean $\pm$ SD of three independent experiments.

10 mmol/l Tris-HCl (pH 9), 0.1% Triton X-100), 25 μ mol/l dNTP's, 100 pmol/l each 5' and 3' primers, 0.37 MBq [α -³²P] dCTP (Amersham, 370 MBq/ml), 5 U Taq polymerase and MgCl₂ to a final concentration of 1.5 mmol/l. PCR products were quantified and normalized to GAPDH using a Bio-Rad Molecular Imager System GS-525 and Molecular Analyst Software (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK).

Northern blot analysis. 30 μ g of RNA was electrophoresed on a 1% agarose-formaldehyde gel prior to transfer to a charged nylon filter (Hybond N⁺, Amersham, UK). Hybridization was done as previously described [11] using cDNA probes to catalase and GAPDH. The membranes were analysed on a Bio-Rad molecular Imager system GS-525 (Bio-Rad) and corrected for the housekeeping gene GAPDH.

Statistical analyses. All statistical analyses were carried out using SPSS and Microsoft excel. Student's *t* tests were used to compare results of experiments unless otherwise stated. Prior to *t* tests, normality of distribution of the data was checked. All results are expressed as means $\pm$ SEM unless otherwise stated, a *p* value of less than 0.05 was considered statistically significant.

Results

The fatty acid profile of cultured VSMC. The effects of the fatty acids are shown in Table 2. The addition

of stearic acid caused a 50% increase in arachidonic acid content of VSMC. Linoleic acid decreased eicosapentanoic acid whereas the addition of α -linolenic acid resulted in an approximate 10-fold increase in eicosapentanoic acid. Lower, more physiological, concentrations for linoleic (0.1 mmol/l) and α -linolenic (0.01 mmol/l) acids were also tested with correspondingly reduced amounts of carrier BSA (0.02 mmol/l and 0.002 mmol/l, respectively); a similar profile was obtained with linoleic acid, the addition of the lower concentration of α -linolenic acid was not associated with a decrease in intracellular arachidonic acid and there was an approximate 25% increase in eicosapentanoic acid in cultured VSMC (results not shown).

Effects of glucose and fatty acids on DNA fragmentation. High glucose alone caused an increase in DNA fragmentation (*p*<0.05). Damage to DNA was increased (*p*<0.05) when polyunsaturated fatty acids were added (0.2 mmol/l) to both glucose conditions (Table 3). Interestingly, despite having an extra double bond, α -linolenic acid was not associated with more DNA damage than linoleic acid. In the lower fatty acid concentration study (Table 3), HG-treated cells sup-

Fig. 1A, B. MDA levels in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid (* p <0.05 vs 5 mmol/l glucose, # p <0.05 vs BSA control, n =8, 5 mmol/l BSA control = 407 ± 89 pmol/mg protein)

Fig. 2A, B. Protein carbonyl levels in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid (* p <0.03 vs 5 mmol/l glucose, # p <0.05 vs BSA control, n =8, 5 mmol/l BSA control = 5.42 ± 1.11 nmol/mg protein)

Table 3. Effects of glucose and fatty acids on DNA damage in VSMC grown in 5 mmol/l (NG) or 25 mmol/l (HG) glucose for 10 days

Treatment	% Tail DNA	
	NG	HG
0.03 mmol/l BSA	9.5 $\pm$ 2.6	18.4 $\pm$ 3.9 ^a
0.03 mmol/l BSA+0.2 mmol/l stearic acid	14.3 $\pm$ 1.1	16.2 $\pm$ 1.0
0.03 mmol/l BSA+0.2 mmol/l oleic acid	8.2 $\pm$ 0.9	12.4 $\pm$ 0.5 ^a
0.03 mmol/l BSA+0.2 mmol/l linoleic acid	37.5 $\pm$ 0.3	50.3 $\pm$ 2.8 ^c
0.03 mmol/l BSA+0.2 mmol/l α -linolenic acid	33.5 $\pm$ 0.1	40.5 $\pm$ 1.4 ^c
0.02 mmol/l BSA	7.4 $\pm$ 1.2	15.4 $\pm$ 0.9 ^a
0.02 mmol/l BSA+0.1 mmol/l linoleic acid	21.7 $\pm$ 1.0	28.9 $\pm$ 1.4 ^c
0.002 mmol/l BSA	6.0 $\pm$ 0.8	15.6 $\pm$ 1.9 ^a
0.002 mmol/l BSA+0.01 mmol/l α -linolenic acid	7.9 $\pm$ 0.3	10.9 $\pm$ 0.9 ^c

Results are expressed as percentage tail DNA, means $\pm$ SE, n =150 from three independent experiments. ^a p <0.05 vs. NG, ^b p <0.02 vs BSA control, ^c p <0.03 vs BSA control

plemented with linoleic acid (0.1 mmol/l) had increased DNA fragmentation (p <0.05) whereas supplementation with more physiological concentrations of α -linolenic acid in 25 mmol/l glucose was associated with reduced DNA damage compared to the relevant control concentrations (p <0.05).

MDA formation, a marker of lipid peroxidation: effects of glucose and fatty acids High glucose conditions

alone caused an increase in cellular MDA (p <0.05). With the exception of oleic acid, MDA concentrations in cells were increased (p <0.05) by supplementation with fatty acids; oleic acid supplementation was associated with a reduction in MDA (Fig. 1A). The MDA content was greatest with α -linolenic acid, followed by linoleic acid and stearic acid. These results indicate that lipid peroxidation alters with the degree of saturation of the fatty acid being tested, producing a J-shaped

Fig. 3A, B. GSH levels in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid (* p <0.04 vs 5 mmol/l glucose within relevant treatment group, # p <0.04 vs basal, 100%=5 mmol/l glucose + BSA control, n =5.5 mmol/l BSA control=2.05 $\pm$ 0.37 nmol/mg protein)

Fig. 4A, B. Glutathione peroxidase activities in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid (# p <0.04 vs BSA control, n =8, 5 mmol/l BSA control = 5.96 $\pm$ 2.80 mU/mg protein)

response. Glucose (25 mmol/l) accentuated the effect of both linoleic and α -linolenic acid on lipid peroxidation. At the lower, more physiological concentration of 0.1 mmol/l linoleic acid caused an increase (p <0.05) in MDA compared to the appropriate glucose/BSA controls (Fig. 1B). By contrast, supplementation with the lower, more physiological concentration of α -linolenic acid (0.01 mmol/l) resulted in a decrease (p <0.05) in MDA content in both glucose conditions, compared to controls (Fig. 1b).

Protein oxidation: effects of glucose and fatty acids. These results follow a similar trend to those for lipid peroxidation (Fig. 2A, B). Glucose alone caused an increase (p <0.03) in protein carbonyl formation in the absence of fatty acids (Fig. 2). Addition of stearic acid caused an increase in protein carbonyl formation compared to controls, as did linoleic acid and α -linolenic acid (p <0.05). In the study with lower concentrations of fatty acid, the addition of linoleic acid (0.1 mmol/l) resulted in an increase (p <0.05) in protein carbonyl formation in both glucose conditions, whereas the addition of α -linolenic acid (0.01 mmol/l) caused a decrease (p <0.05) in protein carbonyl content compared to the BSA controls (Fig. 2B).

Glutathione (GSH) content: effects of glucose and fatty acids. High glucose alone caused a decrease (p <0.04) in cellular GSH content (Fig. 3). The only fatty acid to affect GSH content was α -linolenic acid, which caused an increase (p <0.04) above BSA controls in both glucose conditions (Fig. 3A). At the lower concentrations of linoleic (0.1 mmol/l) and α -linolenic (0.01 mmol/l) acids, linoleic acid again had no specific effect on GSH content, whereas the addition of α -linolenic acid caused an increase (p <0.04) in GSH content (Fig. 3B).

Glutathione peroxidase activity: effects of glucose and fatty acids. High glucose alone did not affect glutathione peroxidase activity (Fig. 4). The addition of α -linolenic acid was associated with a decrease (p <0.04) in enzyme activity in both glucose conditions compared to BSA controls, and at both concentrations of acid used, 0.2 mmol/l and 0.01 mmol/l (Fig. 4).

Glutathione reductase activity: effects of glucose and fatty acids. Glucose (25 mmol/l) alone had no effect on glutathione reductase activity (Fig. 5). However, α -linolenic acid 0.2 mmol/l and 0.01 mmol/l caused a decrease (p <0.03) in enzyme activity in both glucose

Fig. 5A, B. Glutathione reductase activities in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid ($\#p < 0.03$ vs BSA control, $n = 8$, 5 mmol/l BSA control = 11.49 ± 0.95 U/mg protein)

Fig. 6A, B. Catalase activities in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid ($*p < 0.04$ vs 5 mmol/l glucose, $\#p < 0.03$ vs BSA control, $n = 8$, 5 mmol/l BSA control = 8.59 ± 0.78 U/mg protein)

Table 4. The effects of glucose and fatty acids on the gene expression of γ -GCS light and heavy subunits in VSMC

		BSA	18:0	18:1	18:2	18:3
γ -GCS _L	5 mmol/l glucose	100	nd	nd	nd	131.2 ± 9.5^c
γ -GCS _L	25 mmol/l glucose	79.5 ± 1.7^b	74.8 ± 6.0^b	62 ± 1.7^b	78.3 ± 7.3^b	95.0 ± 3.4^c
γ -GCS _H	5 mmol/l glucose	100	nd	nd	nd	127.7 ± 5.8^c
γ -GCS _H	25 mmol/l glucose	79.1 ± 3.4^a	72.8 ± 1.8^a	65.3 ± 17.6^a	78.3 ± 5.2^a	91.4 ± 6.2^c

All results are expressed as a percentage of at least three independent experiments and have been corrected for GAPDH, means $\pm$ SE, ^a $p < 0.05$, ^b $p < 0.02$ vs. NG; ^c $p < 0.05$ vs. relevant glucose/BSA control; nd = not determined

conditions (Fig. 5A,B). Glutathione reductase activity was unaffected by the other fatty acids tested.

γ -GCS subunit gene expression: effects of glucose and fatty acids. Glucose (25 mmol/l) caused a reduction ($p < 0.05$) in gene expression of both the light and heavy subunits of γ -GCS (Table 4). The effects of fatty acids were tested at a single concentration, 0.2 mmol/l. In HG conditions, the addition of stearic, oleic or linoleic acid had no further effect on γ -GCS gene expression. By contrast, addition of α -linolenic acid to HG-treated cells caused increased mRNA expression of both subunits ($p < 0.05$). Furthermore, in the NG conditions, the addition of α -linolenic acid caused an increase ($p < 0.05$) in both light and

heavy subunit gene expression above that of controls (Table 4).

Catalase activity: effects of glucose and fatty acids. Glucose alone had no effect on catalase activity (Fig. 6). However, all of the fatty acids (0.2 mmol/l) increased ($p < 0.03$) catalase activity in both glucose conditions (Fig. 6A). At the lower concentrations, linoleic and α -linolenic acids, also stimulated catalase activity (Fig. 6B).

Catalase gene expression: effects of glucose and fatty acids. Glucose alone had no effect on catalase gene expression however all the fatty acids (0.2 mmol/l) caused a similar increase ($p < 0.05$) in catalase gene expression (Table 5).

Table 5. The effects of glucose and fatty acids on catalase gene expression

	BSA	18.0	18.1	18.2	18.3
5 mmol/l glucose	100	119±4.9	114±9.2	115±3.9	116±4.2
25 mmol/l glucose	103±3.2	128±4.1 ^a	138±6.7 ^a	137±3.1 ^a	130±7.2 ^a

Results are expressed as percentage of basal (5 mmol/l glucose + defatted BSA) and have been corrected for the housekeeping gene, GAPDH; mean ± SE, from four independent experiments; ^a $p < 0.05$ vs controls

Fig. 7A, B. Total superoxide dismutase activities in VSMC with the addition of (A) 0.03 mmol/l BSA and 0.2 mmol/l stearic, oleic, linoleic and α -linolenic acids (B) 0.02 mmol/l BSA and 0.1 mmol/l linoleic acid or 0.002 mmol/l BSA and 0.01 mmol/l α -linolenic acid ($\#p < 0.04$ vs BSA control, $n=8$, 5 mmol/l BSA control = 41.66 ± 0.95 U/mg protein)

Total superoxide dismutase activity: effects of glucose and fatty acids. Glucose alone had no effect on SOD activity (Fig. 7). Stearic acid (0.2 mmol/l) caused an increase ($p < 0.04$) in total SOD activity in both glucose conditions (Fig. 7A). Oleic acid with 25 mmol/l glucose caused a small decrease in SOD compared to both basal and oleic acid with 5 mmol/l glucose (Fig. 7A). The polyunsaturated fatty acids did not affect SOD activity at 0.2 mmol/l (Fig. 7A), or the lower concentrations used for linoleic and α -linolenic acid (Fig. 7B).

Discussion

Diabetes is characterised not only by hyperglycaemia but also by altered metabolism of fatty acids. The fate

of fatty acids is cell specific, with the outcome depending on many variables such as the degree to which fatty acids are involved in oxidative metabolism [24] and in specific gene regulation [10]. Furthermore, the longer term effects of fatty acids, such as in this investigation, depend on the profile of elongase and desaturase enzymes found in each cell type.

In this investigation all of the C18 fatty acids were first used at the same concentration (0.2 mmol/l) with the relevant concentration of defatted albumin, as a carrier, so that simple comparisons of free radical damage could be made. When fatty acids (0.2 mmol/l) were added to cells cultured in normal glucose concentrations there was a J-shaped response with respect to free radical damage with increasing unsaturation of the acid, the nadir was found with oleic acid (18:1). This was most clearly seen with lipid peroxidation but was also present with DNA fragmentation and protein carbonyl formation. These effects were accentuated when the acids were added to higher concentrations of glucose that were capable of causing oxidative stress on their own. For example, although 25 mmol/l glucose alone increased MDA production by about 25% above that seen with 5 mmol/l glucose, addition of the polyunsaturated linoleic and α -linolenic acids to high glucose-treated cells caused a doubling of MDA production. Although less pronounced, a similar phenomenon was seen with both DNA fragmentation and protein carbonyl production.

Why a saturated acid such as stearic acid should be associated with more free radical production than a monounsaturated acid such as oleic acid is unclear. Stearic acid would be expected to be resistant to free radical damage due to the absence of double bonds. However it has been suggested that the chain length of a saturated fatty acid affects its ability to inhibit lipid peroxidation [25]. Saturated fatty acids with chain lengths between 12 and 16 carbons inhibited lipid peroxidation whereas saturates with chain lengths of more than 18 carbons did not inhibit lipid peroxidation. Saturated acids, like other fatty acids, are acted on by desaturase and elongase enzymes; in this project stearic acid was present for a 3-day period, after which there was a substantial increase in the cellular content of arachidonic acid, a polyunsaturated acid that is vulnerable to free radical attack. Furthermore, metabolism of oleic acid led to a reduction in the relative amount of arachidonic acid present in the cells.

The fatty acids increased catalase activity ($p < 0.05$), and this was accompanied by an increase in catalase gene expression ($p < 0.05$). Catalase is produced primarily in the peroxisome, where the metabolism of long chain fatty acids begins. Long chain fatty acids are known to be ligands for peroxisome proliferator-activated receptors (PPARs), which control the expression of peroxisomal genes such as catalase [26, 27, 28].

When linoleic acid was used at a lower, more physiological concentration the effects in VSMC with respect to free radical damage showed a similar profile as the higher concentration, with adjustment for dose. By contrast, the lower, more physiological concentration of α -linolenic acid gave results that were very different from those obtained with the higher concentration. The addition of the lower concentration of α -linolenic acid to 25 mmol/l glucose was associated with reduced DNA fragmentation, MDA production and protein carbonyls compared to 25 mmol/l glucose alone.

The addition of α -linolenic acid was associated with increased GSH content ($p < 0.05$), observed at both concentrations used in this study. Increases in GSH were accompanied by a reduction in glutathione peroxidase activity ($p < 0.04$) and a smaller reduction in glutathione reductase activity ($p < 0.03$). The increase in GSH was probably due to increased gene expression of both the light and heavy subunits of the rate-limiting enzyme in GSH synthesis, γ -GCS. At normal glucose concentrations, the addition of α -linolenic acid was associated with increased expression of γ -GCS mRNA; at high glucose concentrations, addition of α -linolenic acid restored the mRNA expression of both γ -GCS subunits, whereas the other fatty acids had no effect.

The subunits of γ -GCS in the human are coded for by two different genes that are found on different chromosomes. Since VSMC cells were exposed to fatty acids for 3 days, it is not clear whether the increase in γ -GCS gene expression associated with the addition of α -linolenic acid was due to a direct effect of α -linolenic acid itself or to one or more of the longer term changes in fatty acid profile of the VSMC, such as the 10-fold increase in eicosapentanoic acid. Addition of eicosapentanoic acid to VSMC in culture causes an increase in the gene expression of both γ -GCS subunits in high glucose-treated cells (unpublished data). There are many routes by which fatty acids could regulate gene expression, and these might be both tissue and fatty acid specific. In hepatocytes, eicosapentanoic acid seems to be poorly oxidised or incorporated into triglycerides, this could lead to the accumulation of eicosapentanoic acid at a concentration sufficient to activate PPAR nuclear receptors [29]. If fatty acids are metabolised to prostanoids, these could be exported from the cell, bind to G protein-linked membrane receptors to generate second mes-

sengers and, by this means, activate transcription factors [30]. It is not clear by which mechanism α -linolenic acid ultimately caused upregulation of γ -GCS gene expression in VSMC. It is clear, however, that the subsequent increase in GSH content was sufficient to neutralise the potential for free radical damage posed by a combination of high glucose concentration and a physiological concentration of the polyunsaturated α -linolenic acid; by contrast, physiological concentrations of linoleic acid combined with high glucose caused free radical damage to DNA, lipids and proteins in VSMC.

In summary, interactions between fatty acids and glucose can cause oxidative stress in VSMC. However, because the metabolism of fatty acids is tissue specific, and the effects of fatty acids on gene expression are both acid and tissue specific, it is not possible to predict accurately from the degree of unsaturation of the acid its ultimate potential to produce oxidative stress. The results also highlight the important role of endogenous GSH in neutralising free radical activity associated with glucose and fatty acid concentrations that occur in diabetes and the detrimental effect of glucose-induced reduction of γ -GCS gene expression under these conditions.

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