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Decreased aortic glutathione levels may contribute to impaired nitric oxide-induced relaxation in hypercholesterolaemia

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1 The aim of this study was to determine if the decrease in aortic total glutathione (GSH) levels in hypercholesterolaemia is related to the impairment of relaxation to acetylcholine (ACh) and exogenous nitric oxide (NO).

2 Isometric tension and vascular GSH levels were measured in thoracic aortic rings from rabbits fed for 12 weeks with 0.5% cholesterol diet. Hypercholesterolaemia decreased aortic GSH levels and impaired relaxation to ACh and NO.

3 To determine if GSH depletion impaired the response to NO, normal rabbit thoracic aorta was incubated with 1,3-bis [2-chloroethyl]-1-nitrosourea (BCNU; 0.2 mmol L^{-1}), a GSH reductase inhibitor, or diazine-dicarboxylic acid bis [N, N dimethylamide] (diamide; 1 mmol L^{-1}), a thiol oxidizing agent. BCNU or diamide decreased aortic GSH levels and impaired ACh and NO-induced relaxation. The effects of diamide on GSH levels and relaxation were partially prevented by co-incubation with GSH ester (GSE; 2 mmol L^{-1}).

4 Increasing GSH with GSE significantly enhanced NO-induced relaxation in aorta from both hypercholesterolaemic and normal rabbits, however relaxation of hypercholesterolaemic rabbit aorta was not restored to normal.

5 These data suggest that other factors, perhaps related to the long-term decrease in GSH levels, are responsible for reduced NO bioactivity in hypercholesterolaemia.
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Abbreviations: ACh, acetylcholine, BCNU, 1,3-bis [2-chloroethyl]-1-nitrosourea; GSE, glutathione ester; GSH, glutathione; GSNO, S-nitrosoglutathione; GSSG, glutathione disulphide; HC, hypercholesterolaemia; NO, nitric oxide; PE, L-phenylephrine; PSS, physiological salt solution; SNP, sodium nitroprusside

Introduction

Hypercholesterolaemia impairs endothelium-dependent relaxation. This impairment is associated with the development of atherosclerosis (Cohen, 1995), however, its mechanism is not yet clear. Relaxation to authentic nitric oxide (NO) gas in solution is also impaired in hypercholesterolaemic rabbit thoracic aorta. Moreover, cultured smooth muscle cells from hypercholesterolaemic rabbit aorta show reduced responses to NO suggesting that the impairment of vascular function in hypercholesterolaemia may not only be caused by endothelial dysfunction, but also by the reduced response of smooth muscle cells to NO (Weisbrod *et al*., 1997).

Hypercholesterolaemia increases superoxide anion production that can inactivate nitric oxide (Miller *et al*., 1998; Ohara *et al*., 1993). The increase in superoxide anion can generate more reactive free radicals such as hydrogen peroxide or peroxynitrite (Beckman & Koppenol, 1996) that could oxidize proteins and potentially change intracellular calcium homeostasis (Elliott & Koliwad, 1995). These aspects of oxidative stress could contribute to the impaired NO mediated responses in hypercholesterolaemia.

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is the most abundant low molecular weight thiol in vascular tissue (Vita *et al*., 1998), and may affect the bioactivity of NO. S-nitrosothiols may have a role as endothelium-derived relaxing factors (Myers *et al*., 1990; Creager *et al*., 1997), and GSH is the precursor of the nitrosothiol, S-nitrosoglutathione (GSNO). GSNO can transnitrosate protein thiols, possibly changing protein function (Stamler, 1994). GSH is a major antioxidant capable of scavenging hydrogen peroxide, while undergoing oxidation to glutathione disulphide (GSSG), a reaction catalyzed by glutathione peroxidase. Also, GSH scavenges peroxynitrite (Sies *et al*., 1997) and mediates relaxation to peroxynitrite by generating $GSNO_2$ (Balazy *et al*., 1998). Therefore, vascular GSH is an important factor in the bioactivity of NO, and GSH depletion could weaken antioxidant defences against reactive oxygen species and impair NO signalling.

Hypercholesterolaemia reduces vascular GSH levels which impairs the detoxification of peroxynitrite (Ma *et al*., 1997). Oral administration of L-2-oxothiazolidine-4-carboxylic acid, a cell-permeable precursor of GSH, improves endothelial dysfunction in patients with coronary heart disease (Vita *et al*., 1998). Intracoronary infusion of GSH in patients improves vasodilation to acetylcholine (Kugiyama *et al*., 1998). However, the effects and mechanism of GSH supplementation on arterial NO bioactivity in hypercholesterolaemia is not yet clear.

The aim of this study was to determine the relationship between the decreased GSH levels and impaired relaxation to NO in the hypercholesterolaemic rabbit aorta. Also, to study the direct effects of GSH oxidation, normal rabbit aorta was treated with the thiol oxidizing agents, 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU) or diazine-dicarboxylic acid bis[N,N dimethylamide] (diamide) (Becker & Schirmer, 1995; Kosower & Kosower, 1995). Both agents acutely deplete GSH by thiol oxidation. Intracellular GSH was supplemented in rings from

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hypercholesterolaemic rabbits or those treated with the thiol oxidants with GSH ester (GSE), a cell permeable form of GSH.

Methods

Rabbits and diet

Male New Zealand White rabbits were assigned to control or cholesterol fed groups randomly. Control rabbits received a standard diet (Agway Prolab, Agway Syracuse, NY, U.S.A.). Cholesterol-fed animals were fed a diet supplemented with 0.5% cholesterol (w w^{-1}) and 4% peanut oil (w w^{-1}). Cholesterol was dissolved in peanut oil, and the resulting mixture was then added to the standard diet. The diets and water were given *ad libitum* to all animals for 12 weeks (Najibi *et al*., 1994; Najibi & Cohen, 1995; Weisbrod *et al*., 1997).

The rabbits were anaesthetized with sodium phentobarbitone (100 mg kg^{-1}) through a marginal ear vein. After a midsternal thoracotomy, the thoracic aorta was carefully removed and immediately placed in cold physiological salt solution (PSS) of the following composition (in mmol L^{-1}): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, and dextrose 5.5, pH 7.4. Perivascular tissue was carefully removed from the proximal portion of the descending thoracic aorta, which was then cut into 6-mm long ring segments. In the rings on which the relaxation to NO and SNP was to be tested, the endothelium was removed mechanically by placing one tip of a pair of forceps in the lumen and gently rolling the ring 20 to 25 times on a piece of gauze soaked in cold PSS.

Isometric tension

Rings were suspended on triangular stainless steel wires, one stationary and the other connected to a stain gauge transducer (Grass FT03, Quincy, MA, U.S.A.) coupled to a polygraph (Grass7D) to measure circumferential isometric force. Rings were placed in 6-ml organ baths containing PSS that was maintained at 37°C and bubbled continuously with 95% O_2 – 5% CO_2. Rings were stretched in a stepwise fashion to a tension of 6 g, previously shown to be optimal for contraction. The tissues were equilibrated for 90 min, during which the bathing solution was changed every 15 min.

Effect of thiol oxidants on relaxation in normal aortic rings

Some rings from normal rabbits were treated with the thiol oxidant (diamide, 0.2 or 1 mmol L^{-1}, or BCNU, 0.2 mmol L^{-1}) or diamide (1 mmol L^{-1}) with GSE (2 mmol L^{-1}) for 1 h before rings were contracted with L-phenylephrine (PE). Increasing half-logarithmic concentrations of PE (10^{-8}–10^{-5} mol L^{-1}) were added to cause 40–50% maximal contraction, and then rings were relaxed by half-logarithmic increments of acetylcholine (ACh:10^{-9}–10^{-5} mol L^{-1}), sodium nitroprusside (SNP: 10^{-9}–10^{-5} mol L^{-1}) or logarithmic increments of authentic NO gas (10^{-10}–10^{-5} mol L^{-1}). The relaxation was compared with rings from the same rabbits not treated with the oxidants.

Effect of GSE in hypercholesterolaemic rabbit aortic rings

After the initial response to PE and relaxing agent, some rings either from normal or hypercholesterolaemic rabbits were treated with GSE (2 mmol L^{-1}, 1 h) which remained present during a second series of contraction and relaxation. This protocol was chosen when preliminary studies showed that it was necessary for GSE to remain present in order for GSH levels to remain elevated. In addition, preliminary studies showed that there was no difference between the first and second contraction and relaxation without any treatment. The relaxations to ACh, SNP, and NO were compared before GSE treatment. After the second contraction and relaxation, rings were rinsed, and GSH levels were measured. PE, SNP, or NO did not change aortic GSH levels.

GSH measurement

After tension studies, rings were immediately removed and rinsed with PSS (100 ml), blotted, weighed, and homogenized in 0.35 N perchloric acid with 0.5 mmol L^{-1} diethylenetriaminepentaacetic acid (1 : 20 [w v^{-1}]) at 4°C. The homogenate was centrifuged at 14,000 r.p.m. for 15 min at 4°C, and protein-free supernatant was used for measurement. The total level of glutathione (GSH plus GSSG, μmol g^{-1} tissue) was determined by the glutathione reductase enzyme recycling method (Tietze, 1969). Briefly, 100 μl of perchloric acid treated supernatant was mixed with 770 μL of phosphate buffer (0.1 mol L^{-1} NaH_2PO_4, 5 mmol L^{-1} EDTA, pH 7.5), 100 μl of 10 mmol L^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid), and 10 μl of 21 mmol L^{-1} NADPH, and put in a cuvette. The assay was started by adding 1.2 U glutathione reductase (in 20 μl) and the change in absorbance was read at 412 nm for 3 min at 1 min intervals. The absolute values of GSH were determined during each measurement from a standard curve that was made by determining the changes in absorbance that occurred with standard amounts of GSH.

Drugs

GSH reductase and NADPH were purchased from Boehringer Mannheim. NO was made by the following method. Distilled water (750 mL) was placed in a 1 litre i.v. bag with anion exchange resin (Bio-Rad, 200-400 mesh, acetate form) and bubbled with liquid nitrogen gas to remove oxygen. The bag was then filled with NO gas (Matheson Gas Products) and stored in a refrigerator for up to 7 days. The concentration of NO in solution equilibrates to give an approximate 3 mmol L^{-1} saturated solution. The resin binds and eliminates nitrite and nitrate from the solution. Subsequent dilutions are made from this stock by simply drawing off the solution from the bag as required using a syringe (Najibi & Cohen, 1995; Weisbrod *et al*., 1997). All other chemicals were purchased from Sigma.

Data analysis

Relaxation to ACh, NO, and SNP were determined as the maximum relaxation after adding each concentration of drug and calculated as a per cent of the maximal contraction to PE. Data are expressed as mean ± s.e. mean. Statistical evaluation of dose-response curves was performed using an analysis of variance for repeated measures (SAS, Cary, NC, U.S.A.). The difference between groups at individual concentrations, and in aortic GSH levels was tested with Student's *t*-test. Linear regression was calculated

with Microcal Origin 3.5. In all analyses, $P<0.05$ was considered to be statistically significant.

Results

Effect of hypercholesterolaemia on aortic relaxation to ACh, NO, and SNP, and on aortic GSH levels

Hypercholesterolaemia significantly increased the concentration of PE that was needed to contract the aorta either with or without endothelium to a comparable level of tone (Table 1). Hypercholesterolaemia also significantly impaired ACh-, NO-, and SNP- induced aortic relaxation (Figure 1A–C). Hypercholesterolaemia significantly reduced aortic GSH levels to 61% of normal value (Figure 1D).

Effect of BCNU on GSH levels and relaxation of normal aorta to NO

A GSH reductase inhibitor, BCNU (0.2 mmol L^{-1}), significantly decreased vascular GSH levels to 73% of control. Normal rings treated with BCNU required significantly more PE to contract the aorta (Table 1) and significantly impaired NO-induced relaxation (Figure 2).

Effect of diamide on aortic GSH levels and relaxation of normal aorta to ACh, NO, and SNP

Normal rings treated with diamide (1.0 mmol L^{-1}) decreased GSH levels to 37% of control (Figure 3). Diamide (0.2 mmol L^{-1}) did not significantly decrease GSH levels. Co-incubation with GSE (2 mmol L^{-1}) prevented the decrease in aortic GSH levels caused by diamide (1 mmol L^{-1}), and GSH levels were similar to those in rings treated with diamide (0.2 mmol L^{-1}, Figure 3).

Table 1 Contraction to phenylephrine (PE)

	n	*Concentration of PE* (log/mol L^{-1})	*Contraction of PE* (g)
Series 1: (E−)			
Control	8	−7.22±0.08	7.48±0.11
BCNU 0.2 mmol L^{-1}	8	−6.72±0.12*	7.80±0.30
Series 2: (E+)			
Control	8	−6.88±0.04	7.68±0.24
Diamide 0.2 mmol L^{-1}	4	−6.84±0.09	8.00±0.24
Diamide 1 mmol L^{-1}	6	−6.27±0.12*	7.77±0.89
Diamide +GSE	4	−6.86±0.09**	8.40±0.18*
Series 3: (E−)			
Control	16	−7.09±0.07	7.51±0.19
Diamide 0.2 mmol L^{-1}	9	−6.89±0.11	7.49±0.22
Diamide 1 mmol L^{-1}	14	−6.42±0.09*	6.63±0.15*
Diamide +GSE	5	−6.96±0.07	7.64±0.52
Series 4: (E+)			
Normal	6	−6.89±0.04	7.97±0.20
GSE 2 mmol L^{-1}	6	−6.83±0.05	7.90±0.28
HC	10	−6.19±0.11†	6.80±0.22†
HC+GSE 2 mmol L^{-1}	10	−6.03±0.19†	6.60±0.27†
Series 5: (E−)			
Normal	6	−7.11±0.10	7.73±0.21
GSE 2 mmol L^{-1}	6	−6.96±0.03	8.63±0.23
HC	9	−6.74±0.10†	7.38±0.38
HC+GSE 2 mmol L^{-1}	9	−6.45±0.10†,†,†	6.87±0.30†,†,†

Data are mean±s.e.mean of the concentration of phenylephrine used to contract the aorta to approximately 7 g for relaxation studies, as well as the actual contraction attained. The concentration is expressed as the geometric mean of the logarithm of the final concentration used. The contraction is expressed as grams, E+, rings with endothelium; E−, rings without endothelium. *vs control, ** vs diamide, 1 mmol L^{-1}, † vs normal, †† vs HC

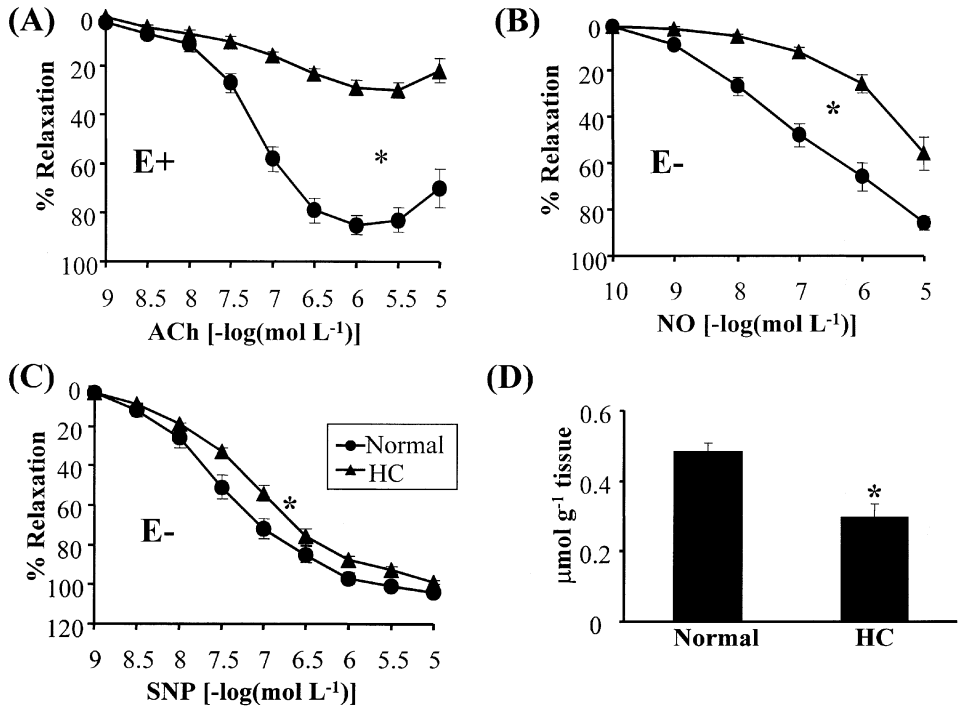

Figure 1 Relaxation to ACh (A), NO (B), and SNP (C), and aortic GSH levels (D) in normal and hypercholesterolaemic rabbit aorta (HC). Relaxation of HC rabbit aorta to ACh ($n=10$), NO ($n=8$), and SNP ($n=8$) were all significantly less than in normal (ACh, $n=10$; NO, $n=14$; SNP, $n=7$). Aortic GSH level (D) in HC rabbit aorta ($n=12$) was also significantly less than in normal aorta ($n=17$). *$P<0.05$ vs normal.

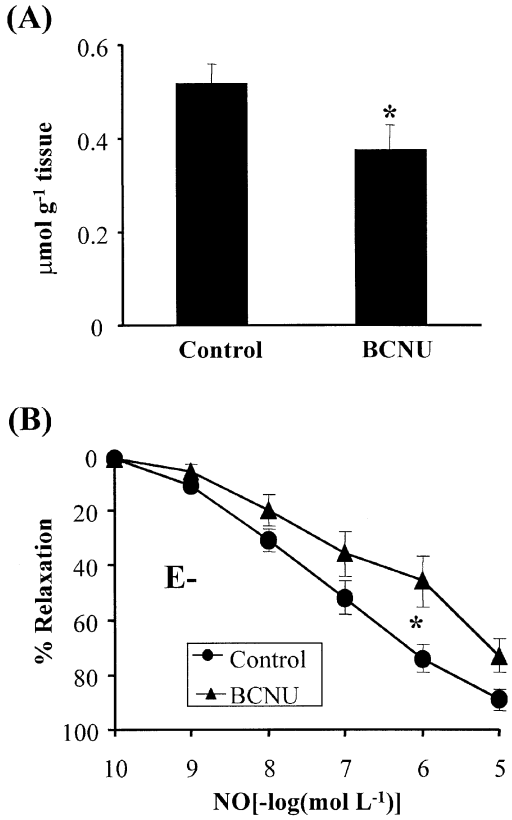

Figure 2 The effect of BCNU (0.2 mmol L^{-1}) on total GSH level (A) and relaxation to NO (B) of normal rabbit aorta. GSH levels were significantly reduced by BCNU ($n=8$, $P<0.05$). Relaxation of aorta treated with BCNU ($n=8$) was significantly less than that of untreated aorta ($n=8$, $P<0.05$).

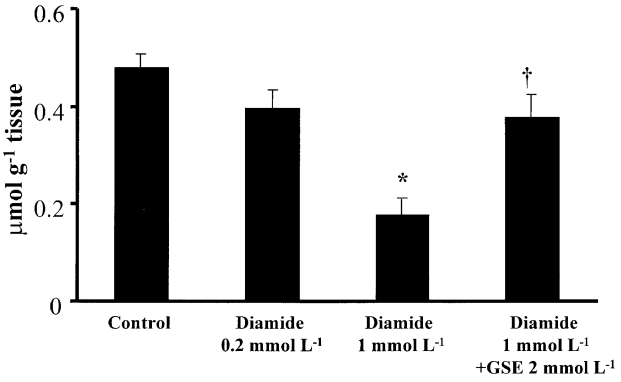

Figure 3 The effect of diamide on aortic GSH levels. Normal aortic rings (control, $n=20$) were treated with either diamide, 0.2 mmol L^{-1}, $n=13$; diamide, 1 mmol L^{-1}, $n=13$; or diamide 1 mmol L^{-1} + GSE 2 mmol L^{-1}, $n=6$; and at the conclusion of tension measurements in aortic rings, GSH was measured. *$P<0.05$ vs control; †$P<0.05$ vs diamide, 1 mmol L^{-1}.

Diamide (1 mmol L^{-1}) increased the dose of PE required to cause similar PE-induced contractions in rings either with or without endothelium, and the co-incubation with GSE restored the contraction (Table 1). Diamide (0.2 mmol L^{-1}) significantly inhibited the relaxation to NO, and diamide (1 mmol L^{-1}) significantly inhibited relaxation to ACh, NO and SNP (Figure 4A–C). Co-incubation of GSE (2 mmol L^{-1}) with diamide (1 mmol L^{-1}) partially restored the relaxation to ACh and NO. The rings treated with the combination relaxed to NO about the same as rings treated with diamide (0.2 mmol L^{-1}, Figure 4B).

Figure 5A–C depicts the relationship between GSH levels of aortic rings treated with diamide and relaxation to ACh (0.1 μ mol L^{-1}, A), NO (0.1 μ mol L^{-1}, B), and SNP (0.1 μ mol L^{-1}, C). There were significant correlations between GSH levels and relaxation to ACh, NO, and SNP.

Effects of GSE incubation on GSH levels and relaxation of normal and hypercholesterolaemic rabbit aorta to ACh, NO and SNP

Figure 6 depicts GSH levels in normal and hypercholesterolaemic rabbit aortic rings with or without incubation of GSE. GSE did not significantly increase GSH levels in normal aortic rings. In aorta from hypercholesterolaemic rabbits, GSH levels were significantly increased by GSE to a level similar to that present in normal aorta not incubated in GSE.

GSE significantly increased the concentration of PE required to contract endothelium-denuded aortic rings from hypercholesterolaemic rabbits, but did not significantly affect

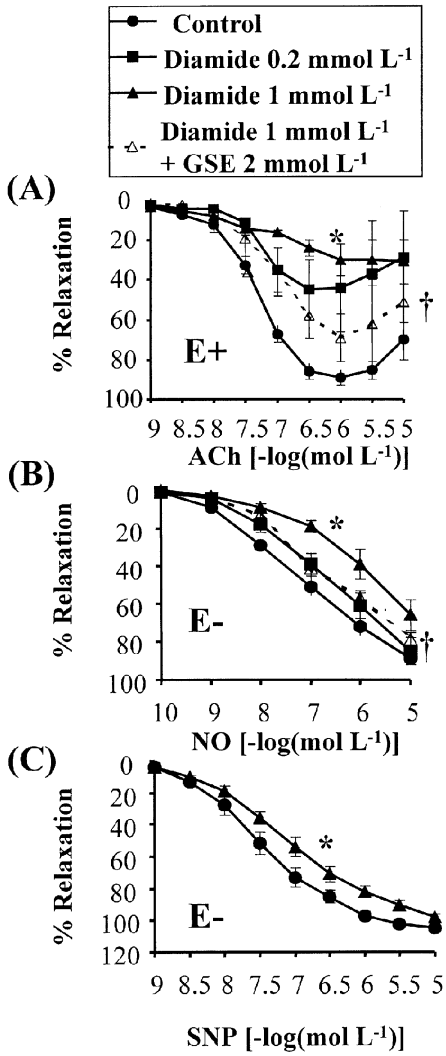

Figure 4 The effect of diamide on relaxation to ACh (A), NO (B), and SNP (C). Normal aortic rings were treated with either diamide (0.2 mmol L^{-1}) or diamide (1 mmol L), or diamide (1 mmol L^{-1}) + GSE (2 mmol L^{-1}). (ACh: control, $n=8$; diamide 0.2 mmol L^{-1}, $n=4$; diamide 1 mmol L^{-1}, $n=6$; diamide 1 mmol L^{-1} + GSE 2 mmol L^{-1}, $n=4$; NO: control, $n=11$; diamide 0.2 mmol L^{-1}, $n=9$; diamide 1 mmol L^{-1}, $n=8$; diamide 1 mmol L^{-1} + GSE 2 mmol L^{-1}, $n=5$; SNP: control, $n=6$; diamide 1 mmol L^{-1}, $n=6$). *$P<0.05$ vs control; †$P<0.05$ vs diamide (1 mmol L^{-1}).

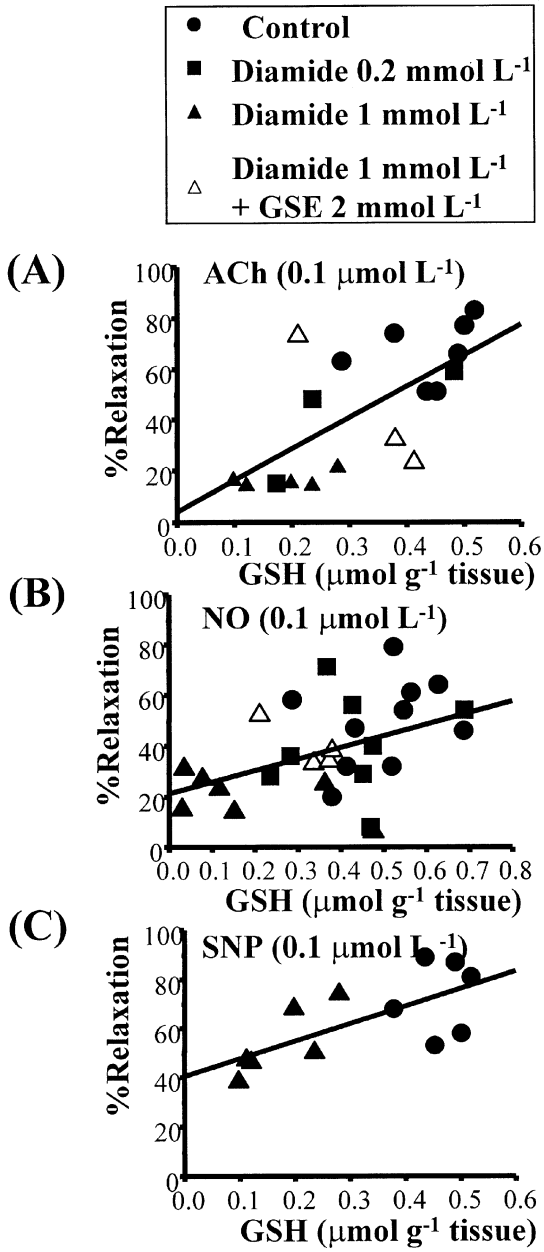

Figure 5 Relationships between aortic GSH levels in rings treated or not with diamide, and relaxation to ACh (A), NO (B), and SNP (C). The data in Figures 3 and 4 were analysed by linear regression. Corresponding values for rings treated with either diamide (0.2 mmol L^{-1}), diamide (1 mmol L^{-1}), or diamide (1 mmol L^{-1})+GSE (2 mmol L^{-1}) and untreated controls were plotted in each graph. Aortic GSH levels and relaxation to ACh (0.1 μmol L^{-1}, $n=18$, $r=0.67$, $P<0.05$), NO (0.1 μmol L^{-1}, $n=29$, $r=0.44$, $P<0.05$), and SNP (0.1 μmol L^{-1}, $n=12$, $r=0.69$, $P<0.05$) was significantly correlated for rings treated or not with diamide with or without GSE.

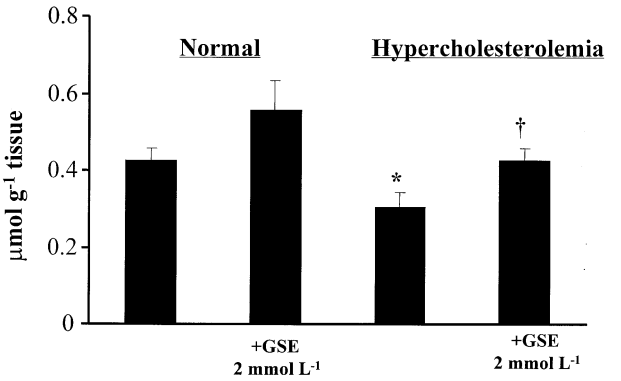

Figure 6 The effect of GSE on aortic GSH levels. Paired rings of normal ($n=8$) and hypercholesterolaemic rabbit aorta ($n=12$) were treated or not with GSE (2 mmol L^{-1}). *$P<0.05$ vs normal without GSE; †$P<0.05$ vs untreated HC.

Discussion

The main findings of this study are that: (1) hypercholesterolaemia decreases aortic GSH levels and reduces relaxations of the rabbit aorta to ACh, NO and SNP, (2) thiol oxidants deplete aortic GSH levels, and reduce relaxation to ACh, NO and SNP, and (3) GSH supplementation prevents the changes caused by thiol oxidants, increases relaxations to NO in both hypercholesterolaemic and normal rabbit aorta, but fails to normalize relaxations to ACh, NO, or SNP.

Numerous mechanisms have been proposed to explain the impairment of endothelium-dependent relaxation in hypercholesterolaemia and atherosclerotic arteries, and an increase in superoxide anion generation is one of the most important factors (Miller *et al*., 1998; Ohara *et al*., 1993). Superoxide anion can inactivate NO and generate peroxynitrite (Beckman & Koppenol, 1996). Superoxide anion is also metabolized to hydrogen peroxide, and these reactive oxygen species may cause oxidative stress damaging cell membranes, proteins, and DNA. The reduction in GSH levels may result from its consumption by detoxifying these reactive oxygen species or from impaired GSH synthesis. Although our study does not clarify the cause of the depletion of aortic GSH, the impairment of relaxation to ACh and NO in hypercholesterolaemia was associated with the reduction in GSH. Therefore, further studies were designed to evaluate if the reduced effectiveness of NO in hypercholesterolaemia is directly related to depletion of GSH.

Thiol oxidants were employed to decrease aortic GSH. Diamide oxidizes GSH, other low molecular weight thiols, and protein thiols. It also reduces free cytosolic GSH by increasing protein binding of GSH (Kosower & Kosower, 1995). When GSH in solution is treated with the same dose of diamide as used in this study, levels of oxidized GSSG are increased, but total GSH levels do not change appreciably (data not shown). This indicates that incubation of the aorta with diamide does not likely affect the GSH measurement. The decreased aortic GSH level after diamide might have been due to an increase in protein-binding of GSH that would not be detected by the assay. BCNU, a GSH reductase inhibitor, increases GSSG levels, and also may increase protein binding of GSH. BCNU also is a thiol-alkylating agent that could by that mechanism decrease aortic GSH levels (Becker & Schirmer, 1995). The correlation between aortic GSH level and relaxation to ACh, NO and SNP of rings treated with diamide alone or

the contractions of normal aortic rings or those with endothelium from hypercholesterolaemic rabbits (Table 1). GSE did not significantly change ACh-induced or SNP-induced relaxation either in normal or hypercholesterolaemic rabbit aortic rings (Figure 7A,C). Incubation with GSE enhanced relaxation to NO of aortic rings from both normal and hypercholesterolaemic rabbits (Figure 7B). After treatment with GSE, NO-induced relaxation of rings from hypercholesterolaemic rabbits remained significantly less than normal.

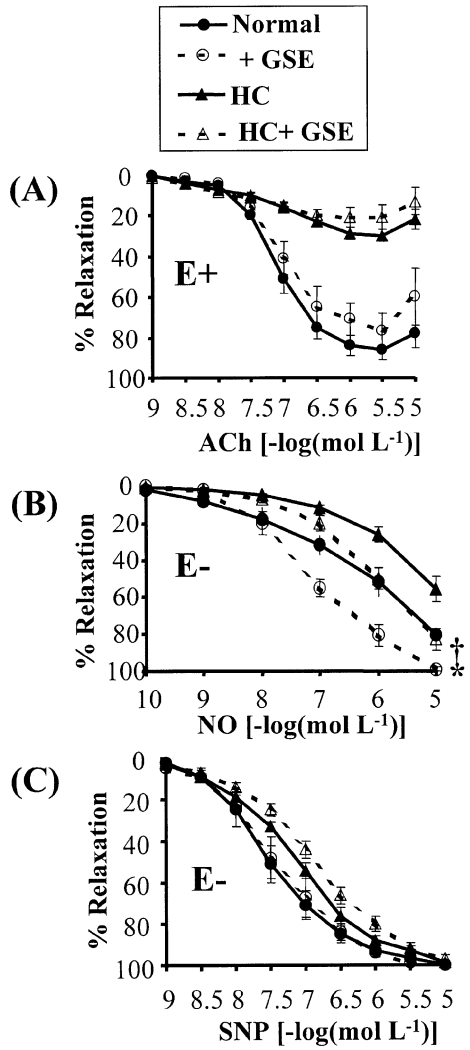

Figure 7 The effect of GSE on relaxation to ACh (A), NO (B), and SNP (C). Aortic rings of normal (ACh, $n=6$; NO, $n=6$; SNP, $n=4$) and hypercholesterolaemic rabbits (ACh, $n=10$; NO, $n=8$; SNP, $n=8$) were treated with or without GSE (2 mmol L^{-1}, 1 h). Relaxation to ACh and SNP were unchanged, but those to NO were increased in both groups. *Indicates the relaxation of normal aorta incubated with GSE is significantly greater than in aorta incubated without GSE, $P<0.05$. †Indicates that the relaxation of hypercholesterolaemic rabbit arteries incubated with GSE is significantly greater than those incubated without GSE, $P<0.05$.

supplemented with GSE suggests the possibility that GSH oxidization is directly related to the decreased relaxation. Based on the relationship between GSH levels and relaxation of normal aortic rings treated with thiol oxidants, we hypothesized that increasing GSH in hypercholesterolaemic rabbit aorta with GSE might improve relaxation.

In preliminary studies to evaluate increasing intracellular GSH, we found that if GSE was removed before contracting and relaxing the aortic rings, GSH levels returned rapidly to the level before treatment, and there were no effects on relaxation. GSE was therefore applied before and during the physiological response. Therefore, it is difficult to exclude the possibility that the effects of GSE on contraction and relaxation of aortic rings were due to extracellular, rather than to intracellular GSH. Nevertheless, aortic GSH levels, determined after extensive rinsing, increased with GSE in the rings from hypercholesterolaemic rabbits to the same level as normal rings without GSE. The result of the attempt to increase aortic GSH levels was to enhance NO-induced relaxation in rings from both normal and hypercholesterolaemic rabbits. This may be due to the fact that in the presence of oxygen, superoxide anion, or trace metals, NO may be oxidized and combine with GSH to form GSNO (Stamler, 1994), and increased formation of this longer lasting form of NO might explain the enhancement in the response observed. If as previously suggested, endothelium-derived NO is itself carried by low molecular weight thiols (Myers *et al*., 1990; Creager *et al*., 1997), this may explain why ACh-induced relaxation was not enhanced by increasing GSH levels. It is not clear why GSH did not change SNP-induced relaxation, but it might be that NO released from SNP does not form nitrosothiols. Because the relaxation to NO was enhanced similarly in both normal and hypercholesterolaemic rabbit aorta, GSE failed to reverse the impairment in relaxation associated with hypercholesterolaemia. It should be noted that the requirement for PE to contract aortic rings in different experimental groups varied, but is unlikely to explain the changes in relaxation observed. For instance, as previously observed (Verbeuren *et al*., 1993), the requirement for PE to contract aorta from hypercholesterolaemic rabbits was increased, and was further increased by GSE. However, hypercholesterolaemia decreased, and GSE increased relaxation to NO. Although other studies have investigated the role of thiol oxidation on endothelium-dependent relaxation, they have emphasized the effects on NO release from the endothelium (Hecker *et al*., 1992; Murphy *et al*., 1991). In contrast, the present studies demonstrate that thiol depletion occurs in, and impairs the response of the smooth muscle.

Factors other than, or additional to the decrease in GSH levels, such as alterations in NO signalling or target proteins in smooth muscle cells might explain impaired relaxation in hypercholesterolaemia. For instance, guanylyl cyclase activity decreases in longer term hypercholesterolaemia (Schmidt *et al*., 1993), making it is possible that reduced NO-stimulated cyclic GMP levels account for the impaired response to ACh and NO. However, it is hard to argue that this is the explanation, because although SNP-induced relaxation was the least affected by hypercholesterolaemia, it is entirely blocked by a guanylyl cyclase inhibitor in both normal and hypercholesterolaemic rabbit aorta (data not shown). In addition, measurements of SNP-stimulated cyclic GMP levels in hypercholesterolaemic rabbit arteries can be normal (Najibi & Cohen, 1995). Authentic NO also can activate cyclic GMP-independent signalling pathways that may involve transnitrosation by nitrosothiols (Stamler, 1994; Weisbrod *et al*., 1998). Therefore, it is possible that cyclic GMP-independent signalling mechanisms involving an interaction of NO or nitrosothiols with target proteins in smooth muscle are impaired during hypercholesterolaemia. These pathways include calcium-dependent potassium channels (Bolotina *et al*., 1994) and the sarcoplasmic reticulum calcium ATPase (Cohen *et al*., 1999), both of which can mediate relaxation to NO. Reactive oxygen species and thiol oxidants may inhibit the function of both these target proteins for NO (Coetzee *et al*., 1995; Scherer & Deamer, 1986; Bolotina *et al*., 1994), so that it is possible that the impaired response to NO caused by diamide or hypercholesterolaemia is due to an oxidant effect on these proteins. If this hypothesis is true for diamide, it would have to affect the proteins in a reversible manner (Kosower & Kosower, 1995), whereas the effect of hypercholesterolaemia is irreversible, at least during short-term treatment with GSE.

Depletion of GSH in hypercholesterolaemic rabbit aorta has been proposed to result in decreased detoxification of

peroxynitrite (Ma *et al*., 1997), and 3-nitrotyrosine present in atherosclerotic arteries suggests that peroxynitrite may be chronically produced (Beckman *et al*., 1994). Peroxynitrite can also irreversibly oxidize thiols (Radi *et al*., 1991), raising the possibility that it is responsible for the impaired relaxation to NO in hypercholesterolaemia. Therefore, the imbalance between an excess of peroxynitrite generation and a decrease in intracellular GSH might be implicated in impaired NO-mediated relaxation in hypercholesterolaemia.

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