British Journal of Pharmacology (1998) 125, 895–901
http://www.stockton-press.co.uk/bjp

Investigation of oxidant stress and vasodepression to glyceryl trinitrate in the obese Zucker rat *in vivo*

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1 We examined the relationship between oxidant stress and the vasodepressor activity of glyceryl trinitrate (GTN) *in vivo*, including rapid GTN tolerance development, in 13-week old obese and age-matched lean Zucker rats which had been maintained for 4 weeks on either control diet or diets enriched with the lipophilic, chain-breaking antioxidants vitamin E (0.5% w w^{-1}) or probucol (0.5% w w^{-1}) or the superoxide anion scavenger tiron (1% w v^{-1} in drinking water).

2 The basal plasma level of the isoprostane 8-epi-$PGF_{2\alpha}$, an *in vivo* marker of lipid peroxidation, was elevated by approximately 5 fold in the obese Zucker rat and markedly reduced by dietary lipophilic antioxidants and depressed by dietary tiron.

3 Vasodepression to bolus does GTN (0.1–100 μg kg^{-1} i.v.), but not endothelium-dependent vasodepression to bolus dose acetylcholine (ACh, 0.02–2.0 μg kg^{-1} i.v.), was impaired in obese animals and completely restored by dietary antioxidants.

4 Nitrate tolerance developed *in vivo* during a 1 h infusion of GTN (40 μg kg^{-1} min^{-1} i.v.) appeared more severe in obese animals. However, rapid nitrate tolerance was not affected by dietary antioxidants in either the obese or lean Zucker rat.

5 We therefore provide evidence that elevated oxidant stress in the obese Zucker rat is associated with an impairment in nitrate vasodepressor activity. However, our data are not consistent with either a role for oxidant stress in rapid nitrate tolerance development in the anaesthetized Zucker rat or the aggravation of this tolerance by pre-existing oxidant stress.

Keywords: Oxidant stress; Zucker rat; nitrate tolerance; nitric oxide

Introduction

Oxidant stress is present in many diseases (see Halliwell, 1991; Maxwell, 1995) including diabetes mellitus (Wolff, 1993; Gopaul *et al*., 1995; Nourooz-Zadeh *et al*., 1995; Giugliano *et al*., 1995a), where it may contribute to diabetic cardiovascular disease (Baynes, 1991; Halliwell, 1993; Giugliano *et al*., 1995a) by impairing endothelial function (Tesfamariam, 1993; Ting *et al*., 1996; Krishna & Das, 1997). The organic nitrates, by providing nitric oxide (NO) (Palmer *et al*., 1987; Moncada, *et al*., 1991), play a central role in the management of coronary artery disease and congestive heart failure (for review, see Ahlner *et al*., 1991); both of which constitute prevalent macrovascular complications in diabetes mellitus (Giugliano *et al*., 1994; Feener & King, 1997).

However, increased levels of reactive oxygen species (ROS) present in diabetic individuals, such as superoxide anion (Paolisso *et al*., 1994; Kawamura *et al*., 1994), have the capacity to diminish the therapeutic action of organic nitrates both by scavenging donated NO (see Darley-Usmar *et al*., 1995; Gryglewski *et al*., 1986; Katusic, 1996) and oxidizing tissue thiols important in nitrate biotransformation (see Chong & Fung, 1990), thereby affording an immediate or 'primary tolerance' (McVeigh *et al*., 1992; 1994). Indeed, oxidant stress may be responsible for the adverse responsiveness to glyceryl trinitrate (GTN) observed by Giugliano *et al*. (1995b) in non-insulin dependent diabetic (NIDDM) patients. In addition to defects in nitrovasodilator reactivity in diabetes mellitus, the clinical potential of organic nitrates is further limited by the development of the more familiar 'secondary tolerance' (Elkayam, 1991; Thadani & de Vane, 1997); a phenomenon which itself may largely be attributable to oxidant stress and in particular, the vascular generation of superoxide anion (Münzel *et al*., 1995; Fink *et al*., 1997; Bassenge & Fink, 1995; Utepbergenov *et al*., 1996; Watanabe *et al*., 1997).

The obese Zucker rat (see Zucker & Antonaides, 1972; Bray, 1977), which is employed in the study of several aspects of NIDDM such as insulin resistance (Stern *et al*., 1972), also exhibits evidence of oxidant stress (Keen *et al*., 1992; Vormann *et al*., 1997) and may thus be a suitable animal model in which to study the relationship between oxidant stress and nitrate action in diabetic man. We therefore verified elevated oxidant stress in the obese animal, using the isoprostane 8-epi-$PGF_{2\alpha}$ as a sensitive *in vivo* marker in plasma (Gopaul *et al*., 1995; Morrow & Roberts, 1996), before proceeding to assess the effects of a number of structurally diverse antioxidants, including the intracellular selective superoxide anion scavenger tiron (Miller & Rapp, 1973; Leffler *et al*., 1991; Münzel *et al*., 1995; Laight *et al*., 1997a), on the vasodepressor activity of GTN. Furthermore, as pre-existing oxidant stress might be expected to result in a more profound tolerance development to organic nitrates (see McVeigh *et al*., 1994; Münzel *et al*., 1995; 1996), we additionally assessed this parameter in a model of rapid GTN tolerance developed in the anaesthetized rat (Newman *et al*., 1990).

Methods

Dietary regimen

Male, 9-week old lean and obese Zucker rats were maintained for 4 weeks on either standard chow (Special Diet Services, Witham, Essex, U.K.) or diet enriched with the lipophilic

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antioxidants probucol or vitamin E ((±)-α-tocopherol acetate) (added to chow each at 0.5% w w^{-1}) or the intracellular superoxide scavenger tiron (added to drinking water at 1% w v^{-1}). Food and water were allowed *ad libitum*.

Vasodepression to ACh and GTN in vivo

Rats were anaesthetized with thiopentone sodium (120 mg kg^{-1} i.p.) and instrumented for the recording of mean arterial pressure (MAP) and the administration of reagents. Following 30 min stabilization, a bolus dose-response curve for the vasodepressor effect of ACh (0.02–2 μg kg^{-1} i.v.) was constructed to assess vasodilator function mediated by endogenous NO. Subsequently, the vasodepressor effect of bolus dose GTN (0.1–100 μg kg^{-1} i.v.) was assessed before and after the rapid induction of nitrate tolerance by a 1 h infusion with GTN (40 μg kg^{-1} min^{-1} i.v.), essentially as described by Newman *et al*. (1990).

Blood collection for analysis of 8-epi-$PGF_{2\alpha}$

In separate groups of animals, 5 ml arterial blood representing a basal sample, was collected in sodium citrate (3.8% w v^{-1}) containing butylated hydroxytoluene (BHT, 20 μM) and indomethacin (15 μM). Blood/citrate was then centrifuged to derive plasma, which was treated with BHT (20 μM) and stored at −80°C prior to analysis. Total plasma 8-epi-$PGF_{2\alpha}$ was measured by gas chromatography-mass spectrometry, as previously described (Gopaul *et al*., 1995).

Materials

(−)Noradrenaline bitartrate, acetylcholine hydrochloride, sodium citrate, butylated hydroxytoluene, indomethacin, tiron (1,2-dihydroxybenzene-3,5-disulphonate), vitamin E ((±)-α-tocopherol acetate) and probucol ([bis(3,5-di-*tert*-butyl-4-hydroxyphenylthio)propane]) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd. (West Drayton, Middlesex, U.K.).

Statistics

Data are expressed as means±s.e.mean. The difference between two means was assessed by Student's unpaired *t*-test while a multicomparison of means was conducted by one way ANOVA followed by Dunnett's test. Vasodepressor responses are plotted as the peak fall in MAP/baseline MAP (%). Comparisons of vasodepressor activity were made using both area under the bolus dose-response curve (AUC) and two way ANOVA. Nitrate tolerance is expressed as tolerant AUC_{GTN}/non-tolerant AUC_{GTN} (%) as previously described (Laight *et al*., 1997b). Statistical significance was accepted at the 5% level.

Results

Body weight, basal MAP and plasma 8-epi-$PGF_{2\alpha}$ levels

Body weight (Table 1) and basal MAP (Table 3) were significantly higher in obese relative to lean rats in all dietary groups. The plasma level of 8-epi-$PGF_{2\alpha}$ was elevated by approximately 5 fold in the obese relative to the lean rat after control diet and was significantly reduced by lipophilic dietary antioxidants and slightly depressed by dietary tiron ($P>0.05$) (Figure 1).

Vasodepressor activity in vivo

Vasodepression to bolus dose ACh (0.02–2.0 μg kg^{-1} i.v.) (Figure 2a) was comparable in lean and obese rats on control diet ($P>0.05$, two way ANOVA), but became greater in obese relative to lean rats after dietary antioxidants (Table 2), which paradoxically depressed lean ACh vasodepressor responses ($P<0.01$, two way ANOVA) (Figure 3a). In contrast, vasodepression to bolus dose GTN (0.1–100 μg kg^{-1} i.v.) in the obese rat was depressed relative to lean rats on control diet ($P<0.01$, two way ANOVA) (Figure 2b and Table 2) without a reduction in potency (obese pED_{50}: 7.64±0.06, $n=6$; lean pED_{50}: 7.66±0.08, $n=6$ ($P>0.05$)). The difference in the vasodepressor action of bolus dose GTN was abolished by dietary antioxidants (Table 2), which selectively enhanced GTN vasodepressor responses in the obese rat, reaching statistical significance with dietary tiron (Figure 4b and Table 2).

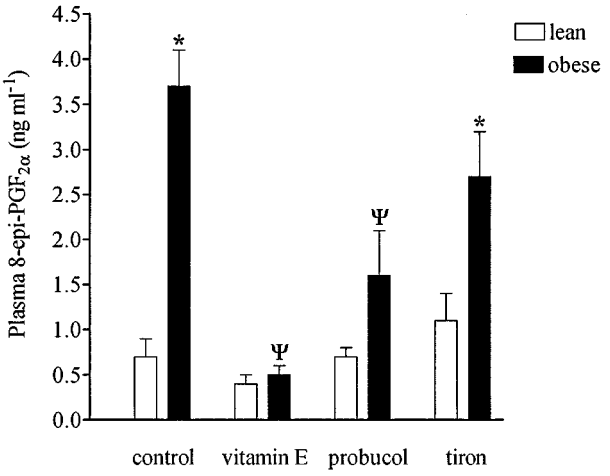

Figure 1 Basal plasma 8-epi-$PGF_{2\alpha}$ levels in 13-week old lean and obese Zucker rats which had been maintained for 4 weeks on control diet or diet enriched with vitamin E (0.5% w w^{-1}), probucol (0.5% w w^{-1}) or tiron (1% w v^{-1} added to drinking water). Values are means±s.e.mean, $n=3-5$. *$P<0.05$ with respect to corresponding control diet value; Ψ$P<0.05$ with respect to obese control diet value.

Table 1 Body weights of 13-week old lean and obese Zucker rats which had been maintained on control or antioxidant diets for 4 weeks

	Lean		*Obese*	
Diet	*Body weight (g)*	n	*Body weight (g)*	n
Control	298.3±9.4	6	473.3±15.7*	6
Vitamin E	325.0±14.3	6	448.3±8.8*	6
Probucol	315.0±6.1	6	485.8±14.3*	6
Tiron	312.0±3.9	5	463.3±16.8*	6

Values are means±s.e.mean. *$P<0.05$ with respect to corresponding lean value.

Rapid nitrate tolerance in vivo

GTN infusion (40 μg kg^{-1} min^{-1} i.v.) elicited a rapid fall in MAP which gradually recovered over the infusion time of 1 h, becoming stable and comparable to the pre-infusion level at 10 min post-infusion (Table 3). Rapid tolerance development to GTN tended to be more severe in obese rats, although statistical significance was not attained (Figure 5 and Table 4).

Table 2 Area under the dose-response curve (AUC) for non-tolerant vasodepression to glyceryl trinitrate (GTN) and acetylcholine (ACh) in lean and obese Zucker rats

	Lean			*Obese*		
Diet	AUC_{ACh} *(units)*	AUC_{GTN} *(units)*	n	AUC_{ACh} *(units)*	AUC_{GTN} *(units)*	n
Control	55.8±2.5	62.4±3.6	6	54.9±3.9	47.6±4.7*	6
Vitamin E	45.7±3.8	51.7±4.4	6	60.9±2.3*	65.0±6.4	6
Probucol	47.4±2.4	59.5±2.1	6	58.2±1.8*	59.8±3.3	6
Tiron	50.2±4.1	59.7±5.8	5	61.8±3.2*	68.5±7.3†	6

Dose-response curves for non-tolerant vasodepressor responses to glyceryl trinitrate (GTN) and acetylcholine (ACh) were constructed in 13-week old anaesthetized lean and obese Zucker rats which had been maintained for 4 weeks on control or antioxidant diets. Values are means±s.e.mean. *$P<0.05$ with respect to corresponding lean value; †$P<0.05$ with respect to control obese value.

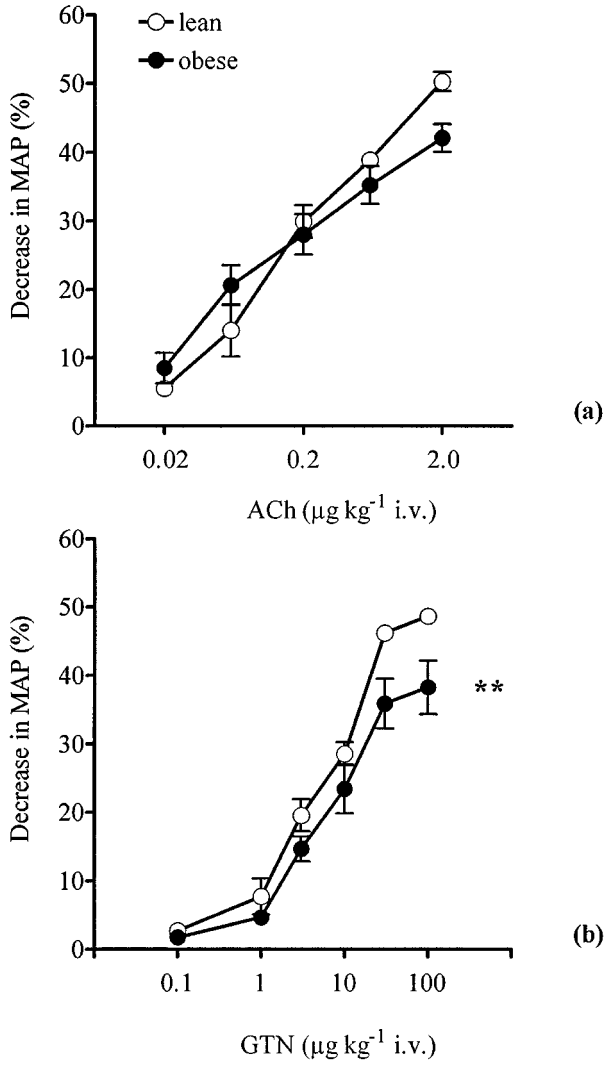

Figure 2 Vasodepressor responses to (a) acetylcholine (ACh) and (b) glyceryl trinitrate (GTN) in non-tolerant, 13-week old lean and obese anaesthetized Zucker rats which had been maintained on control diet for 4 weeks. Values are means±s.e.mean, $n=6$. **$P<0.01$ (two way ANOVA).

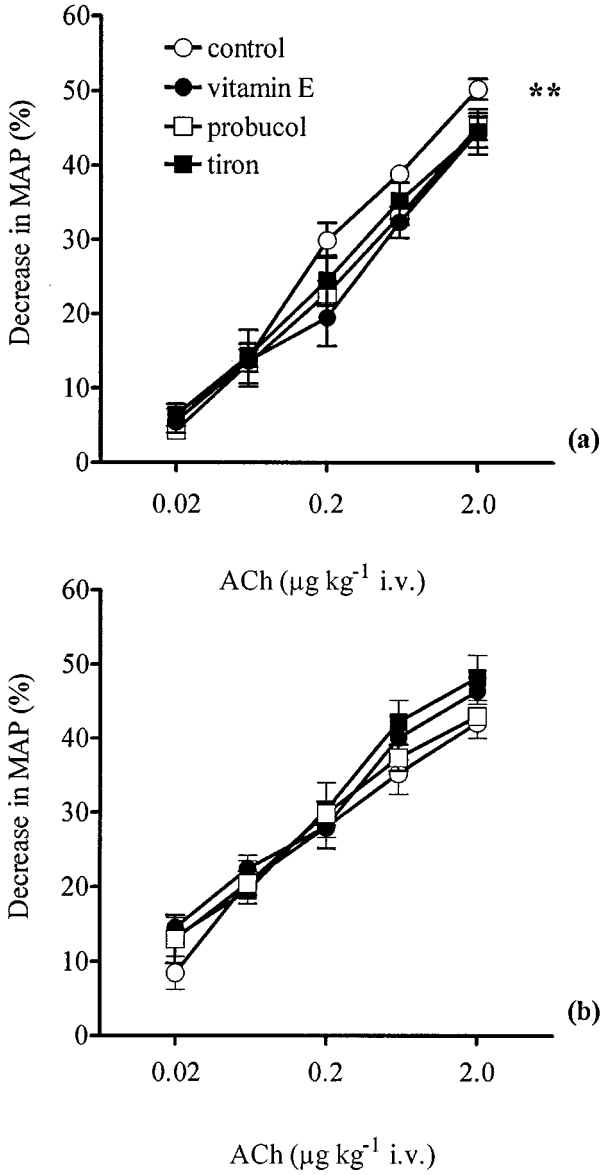

Figure 3 Vasodepressor responses to acetylcholine (ACh) in non-tolerant, 13-week old lean (a) and obese (b) anaesthetized Zucker rats which had been maintained for 4 weeks on control diet or diet enriched with vitamin E (0.5% w w^{-1}), probucol (0.5% w w^{-1}) or tiron (1% w v^{-1} added to drinking water). Values are means± s.e.mean, $n=5-6$. **$P<0.01$ (two way ANOVA).

Table 3 Effects of glyceryl trinitrate (GTN) infusion on mean arterial pressure (MAP) in lean and obese Zucker rats

Diet	*Basal* (mmHg)	*Pre-inf.* (mmHg)	*Lean Max. fall* (%)	*Inf. off* (mmHg)	*Post-inf.* (mmHg)	n	*Basal* (mmHg)	*Pre-inf.* (mmHg)	*Obese Max. fall* (%)	*Inf. off* (mmHg)	*Post-inf.* (mmHg)	n
Control	135.5	134.4	37.7	111.8	121.3	6	153.1*	146.2	32.5	123.3	126.0	6
	(±5.7)	(±4.4)	(±4.0)	(±6.6)	(±6.1)		(±4.6)	(±6.1)	(±3.5)	(±3.1)	(±1.5)	
Vitamin E	119.7	99.5	29.8	105.4	108.7	6	152.1*	147.2*	41.3*	116.1	117.0	6
	(±5.4)	(±18.3)	(±3.4)	(±6.9)	(±7.0)		(±2.9)	(±4.1)	(±1.9)	(±2.7)	(±3.2)	
Probucol	133.2	136.7	34.5	116.4	124.3	6	153.5*	146.0	32.5	126.3	130.9	6
	(±3.9)	(±4.0)	(±3.5)	(±6.2)	(±4.4)		(±1.9)	(±3.3)	(±5.0)	(±2.8)	(±4.0)	
Tiron	126.9	126.8	39.2	108.2	114.2	5	141.5*	138.6	37.2	102.9	118.3	6
	(±3.9)	(±3.7)	(±2.9)	(±4.9)	(±2.3)		(±5.1)	(±4.8)	(±5.8)	(±8.0)	(±6.8)	

MAP was recorded at the onset of the experiment (basal), the beginning (pre-infusion), the end (infusion off) and 10 min following (post-infusion) a 1 h infusion of GTN (40 μg kg^{-1} min^{-1} i.v.) in 13-week old anaesthetized Zucker rats maintained for 4 weeks on control or antioxidant diets. MAP fell rapidly during GTN infusion, reached a minimum (maximal fall) within 2 min of onset, then approached the pre-infusion level. Values are means±s.e.mean. *$P<0.05$ with respect to corresponding lean value.

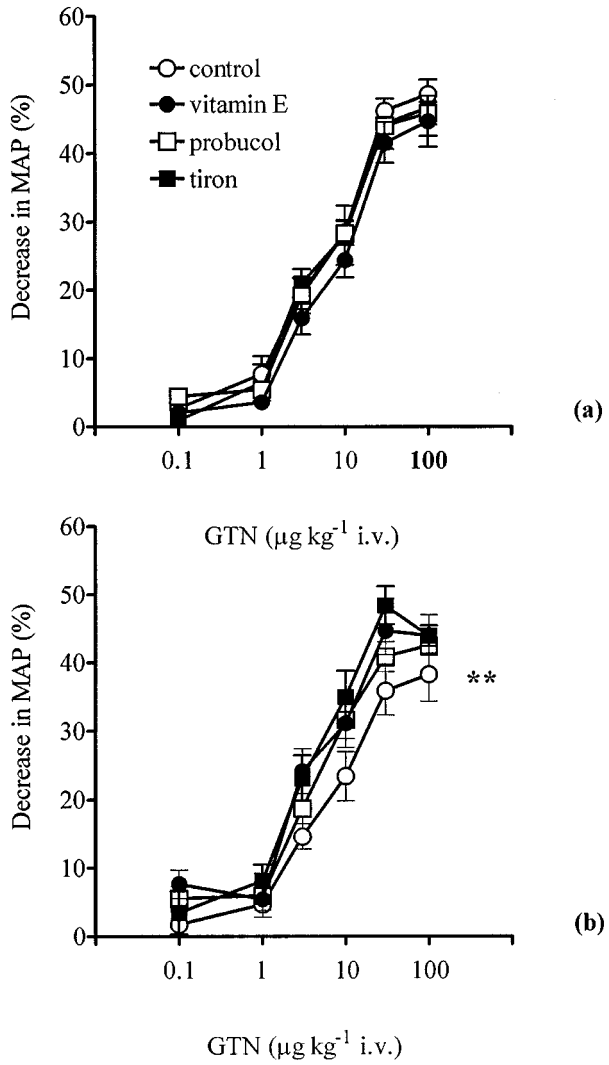

Figure 4 Vasodepressor responses to glyceryl trinitrate (GTN) in non-tolerant, 13-week old lean (a) and obese (b) anaesthetized Zucker rats which had been maintained for 4 weeks on control diet or diet enriched with vitamin E (0.5% w w^{-1}), probucol (0.5% w w^{-1}) or tiron (1% w v^{-1} added to drinking water). Values are means± s.e.mean, $n=5-6$. **$P<0.01$ (two way ANOVA).

Furthermore, rapid nitrate tolerance was not affected by dietary antioxidants in either obese or lean animals (Table 4).

Discussion

In addition to confirming mild hypertension in the 13-week old obese Zucker rat (Ambrozy *et al*., 1991; Pamidimukkala & Jandhyala, 1996), which was unmodified by dietary antioxidants, we report for the first time an impairment in the vasodepressor activity of GTN *in vivo*, pointing to a defect in nitrate-mediated vasodilation at the level of resistance vessels. Furthermore, the enhancement in vasodepression to GTN and subsequent reversibility of this defect in the obese animal by a number of structurally diverse dietary antioxidants, suggests a role for ROS and oxidant stress. In particular, the effectiveness of dietary tiron may indicate the involvement of superoxide anion.

Since vasodepression to ACh was not impaired in the obese Zucker rat, it might be postulated that the selective, antioxidant-sensitive deficiency in nitrate vasoreactivity con-

Table 4 Nitrate tolerance development in lean and obese Zucker rats *in vivo*

	Lean		*Obese*	
Diet	*Tol. AUC_{GTN}/non-tol. AUC_{GTN} (%)*	n	*Tol. AUC_{GTN}/non-tol. AUC_{GTN} (%)*	n
Control	64.7±6.5	6	51.7±6.4	6
Vitamin E	53.8±3.5	6	56.6±12.3	6
Probucol	55.6±5.9	6	57.7±8.3	6
Tiron	63.8±9.8	5	55.8±3.0	6

Tolerance is expressed by the area under the dose-response curve (AUC) for vasodepressor responses to glyceryl trinitrate (GTN), constructed 10 min after the end of a 1 h infusion of GTN (40 μg kg^{-1} min^{-1} i.v.) (i.e. tolerant AUC), as a percentage of non-tolerant AUC in 13-week old anaesthetized lean and obese Zucker rats maintained for 4 weeks on control or antioxidant diets. Low values denote a greater tolerance development. (Values are means±s.e.mean).

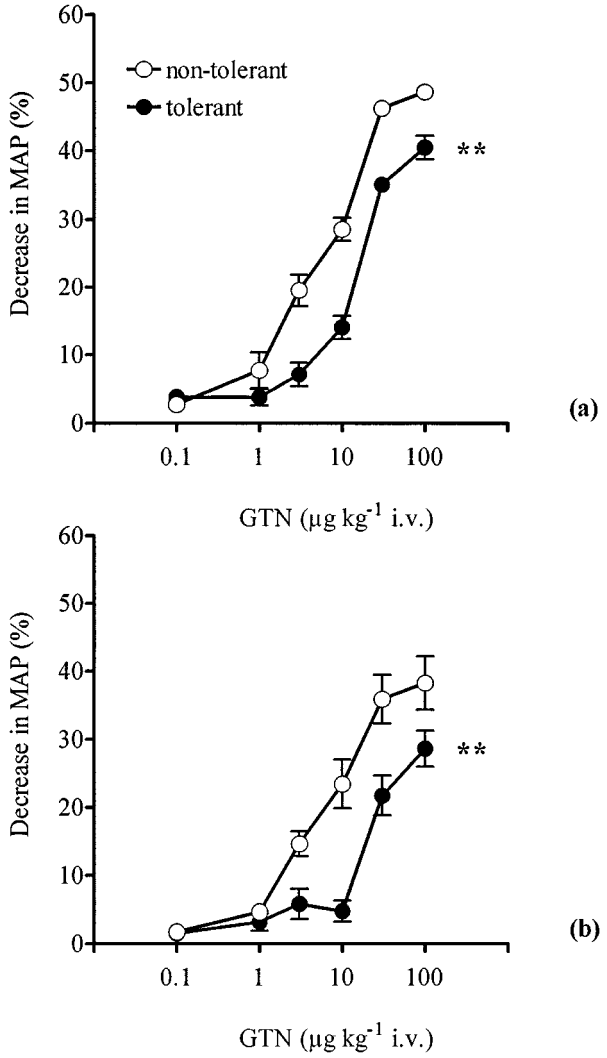

Figure 5 Vasodepressor responses to glyceryl trinitrate (GTN) before and 10 min after the end of 1 h GTN infusion (40 μg kg^{-1} min^{-1} i.v.) in 13-week old lean (a) and obese (b) anaesthetized Zucker rats which had been maintained for 4 weeks on control diet. Values are means±s.e.mean, $n=5-6$. $^{**}P<0.01$ (two way ANOVA).

cerns the oxidation of tissue thiols important in organic nitrate biotransformation and/or the inhibition of organic nitrate converting enzyme(s) (ONCE) (see Chong & Fung, 1990; Bennett *et al*., 1994; McVeigh *et al*., 1994; Laight *et al*., 1997b). However, the finding that the potency of GTN-mediated vasodepression was not reduced in obese relative to lean animals, clearly does not support this notion. An alternative explanation for the differential pattern of impairment is that nitrate-derived NO is in some way more susceptible to inactivation by superoxide anion, which seems unlikely. The reason(s) for the preservation of ACh-mediated vasodepression in the obese Zucker rat, in the face of a superoxide anion-mediated impairment in NO-dependent vasodilation, therefore remains unclear. However, it is tempting to speculate that our observations may reflect an enhancement in endothelial function in the young obese Zucker rat (see Auguet *et al*., 1989; Sexl *et al*., 1995; Growcott *et al*., 1995; Turner & White, 1996), which then compensates for the vascular 'NO resistance' mediated by superoxide anion. Indeed, this could conceivably represent an adaptation to oxidant stress in this animal (see Keen *et al*., 1992; this report).

It is interesting to note that while dietary vitamin E, probucol and the hydrophilic superoxide anion scavenger tiron all showed comparable efficacy in restoring bolus GTN vasodepressor activity in the obese Zucker rat to the corresponding level in lean animals, this effect was only accompanied by significant, marked falls in obese plasma 8-epi-$PGF_{2\alpha}$ in the case of lipophilic antioxidants. This may suggest that while plasma 8-epi-$PGF_{2\alpha}$ is a useful marker of oxidant stress and lipophilic antioxidant action in particular (see Gopaul *et al*., 1995), an inhibition of lipid peroxidation in the plasma compartment is probably unrelated to the modalities and/or site of antioxidant action benefiting nitrate vasoactivity in the obese Zucker rat *in vivo*.

Oxidant stress secondary to chronic nitrate exposure has been implicated in clinically relevant models of nitrate tolerance (Bassenge & Fink, 1995; Münzel *et al*., 1995; Watanabe *et al*., 1997). In the present study, the failure of dietary antioxidants to affect rapid tolerance development to high dose GTN in either the anaesthetized lean or obese Zucker rat therefore probably reflects differences in experimental tolerance induction. N-acetyl-L-cysteine prevents rapid nitrate tolerance in the rat *in vivo* (Newman *et al*., 1990), suggesting that a defect in enzyme-dependent nitrate biotransformation may be more important in this pharmacological model. Furthermore, it is clear that pre-existing oxidant stress in the obese Zucker rat, reflected by raised basal plasma isoprostane levels, could not be associated with the apparently more severe rapid nitrate tolerance observed in this animal *in vivo* (see McVeigh *et al*., 1994; Giugliano *et al*., 1995b).

In conclusion, the evidence of raised oxidant stress and antioxidant-sensitive GTN vascular dysfunction in the obese Zucker rat *in vivo* is consistent with the notion that primary nitrate action is sensitive to disruption by ROS in diabetes mellitus (McVeigh *et al*., 1994; Giugliano *et al*., 1995b). The obese Zucker rat may therefore provide a valuable research model in which to further investigate the relationship between oxidant stress and obesity- and NIDDM-related pathologies.

This work was supported by Lipha s.a., Lyon, France.

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(Received May 15, 1998
Revised July 16, 1998
Accepted July 22, 1998)