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Endothelin Receptor Antagonism Does Not Prevent the Development of In Vivo Glyceryl Trinitrate Tolerance in the Rat[1]

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ABSTRACT

There is evidence that increased endothelial production of endothelin-1 (ET-1) may contribute to glyceryl trinitrate (GTN) tolerance. We used the competitive ET_A receptor antagonist ZD2574 to determine whether chronic ET_A receptor blockade affected the biochemical and functional responses to GTN during the development of GTN tolerance in vivo. Tolerance induced using transdermal GTN patches resulted in a 5.3 ± 1.2-fold increase in the EC_{50} value for GTN relaxation in isolated aorta from GTN-tolerant rats. Coadministration of ZD2574 (100 mg kg^{-1} t.i.d. for 3 days) during tolerance induction had no effect on GTN-induced relaxation. This dose of ZD2574 markedly blunted the pressor response to ET-1, indicating effective blockade of ET_A receptors, and also abolished the initial transient depressor response to ET-1, indicating that blockade of endothelial ET_B receptors also occurred using this dosage regimen for ZD2574. Consistent with the relaxation data, coadministration of ZD2574 had no effect on the decrease in GTN-induced cGMP accumulation or on the decrease in GTN biotransformation that occurred in aortae from GTN-tolerant animals. Radioimmunoassay data indicated that the GTN tolerance induction protocol caused a 2.3 ± 0.4-fold and a 2.2 ± 0.5-fold increase in total tissue ET-1 levels in tolerant aorta and vena cava, respectively. These data suggest that chronic inhibition of ET receptors by ZD2574 was not sufficient to prevent or diminish the tolerance-inducing effects of GTN, and that the increase in ET-1 levels observed in tolerant tissues may occur as a consequence of the vascular changes that occur during chronic GTN exposure.

Tolerance to the hemodynamic and antianginal actions of organic nitrates has been problematic with respect to their clinical effectiveness during long-term treatment. Several vascular mechanisms have been suggested to explain the development of nitrate tolerance after chronic glyceryl trinitrate (GTN) exposure. These include depletion of critical sulfhydryl groups (Needleman and Johnson, 1973), diminished activity of soluble guanylyl cyclase or increased activity of phosphodiesterase enzymes (Axelsson and Andersson, 1983; Waldman et al., 1986), reduced biotransformation of GTN to nitric oxide (NO) (Brien et al., 1988), and increased production of superoxide anion (Münzel et al., 1995b, 2000). Evidence exists to both support and refute many of these mechanisms, suggesting that the development of tolerance to the therapeutic effects of GTN is complex and multifactorial.

GTN is considered to act as a prodrug, in that it requires biotransformation to its active metabolite (NO or a closely related species) before initiating its pharmacological effect of activation of soluble guanylyl cyclase and the accumulation of intracellular cGMP within the vascular smooth muscle cell. A number of studies have examined the enzyme systems thought to be involved in the biotransformation of organic nitrates to NO, including the glutathione *S*-transferases (Nigam et al., 1996) and the cytochrome P450 system (McDonald and Bennett, 1993; McGuire et al., 1994, 1998), and it has been shown that tolerance is associated with decreased biotransformation of organic nitrates (Brien et al., 1986; Bennett et al., 1989; Slack et al., 1989; Stewart et al., 1989).

More recent evidence regarding GTN tolerance suggests that there may be an autocrine component, whereby increased endothelial production of endothelin-1 (ET-1) may contribute to tolerance by enhancing vasoconstriction through a protein kinase C (PKC)-dependent mechanism (Münzel et al., 1995a), or by increasing vascular superoxide production (Kurz et al., 1999). It has also been reported that the in vivo coadministration of the PKC inhibitor *N*-benzoylstaurosporine was able to prevent the development of vascular tolerance as assessed by the ex vivo responses of isolated rat aorta to GTN (Zierhut and Ball, 1996). Significant in-

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ABBREVIATIONS: GTN, glyceryl trinitrate; NO, nitric oxide; ET-1, endothelin-1; PKC, protein kinase C; Ang II, angiotensin II; 1,2-GDN, glyceryl-1,2-dinitrate; 1,3-GDN, glyceryl-1,3-dinitrate; ZD2574, 2-(4-isobutylphenyl)-*N*-(3-methoxy-5-methylpyrazin-2-yl)-pyridine-3-sulfonamide; AUC, area under the curve; MAP, mean arterial pressure.

creases in vascular levels of ET-1 during the development of GTN tolerance could be expected to increase vasoconstrictor tone in the peripheral circulation and oppose the beneficial vasodilatory effects of GTN. ET-1 has been reported to play an important role in endothelial regulation of vascular tone and has been implicated in contributing to several pathophysiological conditions, including congestive heart failure (Haynes and Webb, 1998). Vasoconstriction by ET-1 occurs after its binding primarily to ET_A receptors on vascular smooth muscle cells, resulting in the G-protein-dependent activation of phospholipase C_β and the subsequent formation of inositol triphosphate and activation of PKC. PKC activation can occur in response to several other vasoactive substances [e.g., angiotensin II (Ang II), vasopressin, norepinephrine], which may also be affected during the development of GTN tolerance.

The purpose of this study was to investigate the role, if any, that ET-1 plays in the development of GTN tolerance. The competitive ET_A receptor antagonist ZD2574 was used to determine whether chronic ET_A receptor blockade had any effect on the biochemical and functional responses to GTN during the in vivo development of GTN tolerance.

Materials and Methods

Drugs and Solutions. Krebs' solution was composed of the following: 118 mM NaCl, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 2.5 mM $CaCl_2$, 24.9 mM $NaHCO_3$, and 10 mM glucose. The solution was aerated with 95% O_2, 5% CO_2 and maintained at 37°C. Transdermal GTN patches were obtained as Transderm-Nitro brand (0.2 mg h^{-1}) from CIBA Pharmaceuticals (Mississauga, Ontario, Canada). Drug-free (sham) patches were produced by soaking the patches for a minimum of 2 days in 95% ethanol (patches were allowed to air dry for 30 min before implantation). GTN was obtained as a solution (Tridil, 5 mg ml^{-1}) in ethanol, propylene glycol, and water (1:1:1.33) from DuPont Pharmaceuticals (Scarborough, Ontario, Canada). Glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) were prepared by acid hydrolysis and purified by thin-layer chromatography (Brien et al., 1986). The concentrations of GTN, 1,2-GDN, and 1,3-GDN in stock solutions were determined by a spectrophotometric method as described previously (Bennett et al., 1988). L-Isoidide dinitrate was obtained from D. H. Stereochemical Consulting Ltd. (Vancouver, British Columbia, Canada). Stock solutions of isoidide dinitrate were prepared by extraction of organic nitrate-lactose powder (50% w/w) with ethanol. Further dilutions were made with the appropriate buffer solution. Isosorbide-2-mononitrate was a gift from Wyeth Ltd. (Toronto, Ontario, Canada). ZD2574 [2-(4-isobutylphenyl)-*N*-(3-methoxy-5-methylpyrazin-2-yl)-pyridine-3-sulfonamide; mol. wt. 412.5] was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). The following items were purchased for the ET-1 radioimmunoassay: rabbit anti-ET-1 serum and ET-1 (Peninsula Laboratories Inc., Belmont, CA), goat anti-rabbit IgG and normal rabbit serum (Immunocorp, Montreal, Quebec, Canada), and ^{125}I-Tyr^{13}-ET-1 (Mandel Scientific, Guelph, Ontario, Canada). L-Phenylephrine hydrochloride, porcine ET-1, Triton X-100, polyethylene glycol-8000, and heparin sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of at least reagent grade and were obtained from a variety of sources.

Induction of GTN Tolerance In Vivo. Studies used 250- to 300-g male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada). GTN-tolerance was induced by exposing rats to a continuous source of GTN via the subdermal implantation of two 0.2 mg h^{-1} transdermal GTN patches (tolerant) or drug-free patches (control) for 48 h. To implant the patches, rats were anesthetized with halothane. A small area was shaved in the upper dorsal region and the site was disinfected with 2.5% iodine. A 1-cm transverse incision was made and the skin was separated from the underlying fascia by blunt dissection. Two transdermal patches were inserted back-to-back into the resulting subdermal space. The site was sutured closed and disinfected again with iodine. At 24 h, the site was reopened and both patches were replaced. At 48 h, the animals were used for the following in vitro or in vivo studies.

ZD2574 Administration Protocol for In Vitro Studies. To assess the effects of ET_A receptor blockade, ZD2574 (100 mg kg^{-1}) was administered t.i.d. by i.p. injection for 3 days. Control rats were administered the equivalent volume of drug vehicle (dimethyl sulfoxide, 1 ml kg^{-1}). Previous dose-response data indicated that this dose of ZD2574 had a maximal inhibitory effect on the pressor response to an i.v. bolus of ET-1 over an 8-h period. There were four treatment groups in all: control-vehicle-treated, control-ZD2574-treated, tolerant-vehicle-treated, and tolerant-ZD2574-treated. ZD2574 treatment was initiated 24 h before the GTN patch implantation surgery and continued for the duration of the tolerance induction protocol. In the in vitro studies using isolated rat aorta, animals received the final dose of ZD2574 1 h before tissue harvest.

Relaxation Studies. Endothelium-intact isolated thoracic aortic strips were prepared for isometric tension measurements as described in Stewart et al. (1989). Tissues were contracted maximally with 10 μM phenylephrine to ensure the viability of the preparation. After a 30-min washout period, the tissues were contracted submaximally with 0.1 μM phenylephrine. Once the induced tone had stabilized, cumulative concentration-response curves to GTN (0.1 nM–10 μM) were obtained. Tissue relaxation to GTN was measured as the percentage decrease in phenylephrine-induced tone.

In another study, the in vitro responses to ET-1 were assessed in aorta from tolerant and ZD2574-treated animals, and in tissues exposed to ZD2574 in vitro. Cumulative concentration-response curves to ET-1 (0.1 nM–0.1 μM) were obtained in the presence or absence of 500 nM ZD2574. Previous in vitro concentration-response data indicated that this concentration of ZD2574 was sufficient to completely block ET-1-induced contractions. Tissue contraction to ET-1 was measured as a percentage of the maximum ET-1-induced tone in untreated aorta.

Tissue Biotransformation Studies and cGMP Determination. Aortae were divided in thirds and placed into individual tubes containing Krebs' solution at 37°C aerated with 95% O_2, 5% CO_2. Two segments were used for cGMP determinations and the other for assessing GTN biotransformation. To assess GTN biotransformation, tissues were exposed to 0.1 μM phenylephrine for 10 min, followed by 2 μM GTN for 1 min, and then frozen between liquid nitrogen precooled clamps. The 1,2-GDN and 1,3-GDN metabolites of GTN were extracted from the tissues as described in Bennett et al. (1992) and were quantitated by megabore capillary column gas-liquid chromatography as described in McDonald and Bennett (1990). The tissues were digested with 2 N NaOH (1 ml) for 48 h and aortic protein levels determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

For cGMP determinations, tissue segments were exposed to 0.1 μM phenylephrine for 10 min followed by either 2 μM GTN for 1 min or an equal volume of Krebs' solution (basal). At the end of the incubation period, all tissue segments were frozen between liquid nitrogen precooled clamps. Tissues were homogenized in 1 ml of 6% trichloroacetic acid and centrifuged at low speed for 20 min. The supernatant fractions were extracted six times with 2 ml of water-saturated diethyl ether, acetylated, and cGMP was quantitated by radioimmunoassay. The pellet was digested in 2 N NaOH for 48 h and aortic protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Surgical Preparation for Hemodynamic Studies. Male Sprague-Dawley rats (250–275 g) had catheters (30-gauge Teflon fused to 0.02-inch inside diameter Tygon) inserted into the abdominal vena cava (one or two catheters), the abdominal aorta, and/or the i.p. cavity. The animals were anesthetized for surgery with a combi-

nation of ketamine (Rogarsetic, 70 mg kg^{-1} i.p.) and xylazine (Rompun, 5 mg kg^{-1} i.p.). The catheters were externalized between the scapulae, sutured into position, and filled with 50 I.U. ml^{-1} heparin saline to help maintain patency during the ensuing 72-h postsurgery recovery period.

Effect of ZD2574 on ET-1-Mediated Changes in Blood Pressure. To determine the efficacy of the in vivo ZD2574 dosage regimen, the blood pressure response to a 1-min infusion of ET-1 (2500 ng kg^{-1}) was measured every 2 h over an 8-h period. This high dose of ET-1 was chosen because we wanted to ensure that the dose of ZD2574 was sufficient to block the ET_A receptor-mediated effects of the high concentrations of ET-1 that may be expected due to its local, abluminal release within the vasculature (Wagner et al., 1992). For the 24-h period before blood pressure measurements, ZD2574-treated rats were given an i.p. dose of 100 mg kg^{-1} (dissolved in dimethyl sulfoxide, 100 mg ml^{-1}) and control rats were given the equivalent volume of drug vehicle every 8 h (four doses in all). Baseline recording of mean arterial pressure (MAP) commenced 1 h after the final ZD2574/vehicle dose so that at the 2-h time point after the last dose of ZD2574 the first ET-1 infusion was administered and the change in MAP recorded. Continuous blood pressure and heart rate measurements were made on conscious, unrestrained rats by connecting the aortic catheter to a Cobe (model CDX3) pressure transducer coupled to an ETH-400 transducer amplifier and MacLab data acquisition system (A. D. Instruments, Milford, MA). Once a stable baseline pressure was established and the animals had adjusted to the recording conditions, the following experimental protocol was followed. Baseline MAP measurements were collected for a minimum of 30 min. The increase in MAP due to the 1-min ET-1 infusion was assessed by comparing the area under the curve (AUC) between the ZD2574-treated and vehicle-treated animals for a 90-min period after the ET-1 infusion was started. This was repeated every 2 h after the last dose of ZD2574 for a total of four measurements over an 8-h period. We also assessed the effect of ZD2574 on the initial vasodilator response to ET-1, which is due to NO release from endothelial cells mediated by ET_B receptors.

Radioimmunoassay for Tissue Levels of ET-1. The levels of ET-1 in control and tolerant aortic and vena caval tissues were measured by radioimmunoassay after acid extraction of ET-1 from the tissue samples. The entire thoracic aorta and vena cava were removed from sham-treated and GTN-tolerant rats, cleaned, and immediately frozen in liquid nitrogen. Each tissue was weighed and vena cava samples were pooled (four or five) to obtain sufficient starting material for analysis. The ET-1 was extracted using 20 μl of an ice-cold extraction buffer (1% w/v NaCl, 1 N HCl, 1% w/v formic acid, 1% w/v trifluoroacetic acid) per milligram of frozen tissue. Each tissue sample was homogenized in buffer with a Polytron at 30,000 rpm for 15 to 30 s and centrifuged for 30 min at 5000*g* at 4°C. The supernatant was divided into aliquots, frozen in liquid nitrogen, and stored at −80°C for analysis the following day.

To purify the ET-1, a C18 SepPak column was prepared by washing with 4 × 5 ml of methanol, followed by 4 × 5 ml of acetonitrile, and then 4 × 5 ml of buffer A (0.1% v/v trifluoroacetic acid). The supernatant was thawed on ice, mixed with an equal volume of 2× buffer A, and transferred to the column. The column was immediately washed four times with 5 ml of buffer A, followed by 3.5 ml of 20% v/v acetonitrile, 0.1% v/v trifluoroacetic acid. ET-1 was eluted with 3.5 ml of 50% v/v of acetonitrile, 0.1% trifluoroacetic acid. The solvent was evaporated under nitrogen gas until 0.5 ml remained. The sample was frozen in liquid nitrogen and allowed to freeze dry overnight to remove the remaining solvent. The next day, the sample was resuspended in 0.1 ml of buffer A and the volume made up to 0.5 ml using freshly prepared radioimmunoassay buffer that consisted of 100 mM sodium phosphate (pH 7.4), 50 mM NaCl, 0.01% w/v NaN_3, 0.1% w/v bovine serum albumin, and 0.1% v/v Triton X-100.

Each 0.5-ml sample was assayed by adding 0.1 ml of radioimmunoassay buffer, 0.1 ml of rabbit anti-ET-1 antibody (dilution 1:2.5), and 0.1 ml of sample or standard. This incubation mixture was vortexed and allowed to equilibrate for 18 to 24 h at 4°C. On day 2, 0.1 ml of ^{125}I-Tyr^{13}-ET-1 (6000 cpm) was added to all samples, vortexed, and allowed to equilibrate for another 18 to 24 h at 4°C. On day 3, 0.1 ml of goat anti-rabbit IgG (dilution 1:25) and 0.1 ml of normal rabbit serum (dilution 1:50) were added to all samples except the total counts. The samples were vortexed and allowed to incubate at room temperature for 2 h before adding 0.1 ml of polyethylene glycol-8000 (25% w/v). The samples were vortexed and left for 15 min. Finally, each sample was vortexed with 0.5 ml of radioimmunoassay buffer and centrifuged at 7000*g* for 20 min. The supernatant was removed from each sample and the pellet counted for 1 min using a Beckman 4000 gamma counter. ET-1 standards were prepared for the radioimmunoassay in the range of 0 to 1024 pg. The limit of detection for this assay was 1 pg of ET-1.

Data Analysis. All data are presented as the mean ± S.D. EC_{50} values were determined from each concentration-response curve by interpolation. Unless indicated otherwise, data from all experiments were analyzed by a one-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. The assumption of homogeneity of variance was tested in all cases using Bartlett's test. Due to inhomogeneity of variance, statistical analysis for the relaxation experiments was performed using logarithmically transformed data. A P value of .05 or less was considered statistically significant.

Results

Relaxation Studies. In a preliminary set of control experiments it was shown that the in vivo treatment of rats with ZD2574 (100 mg kg^{-1} t.i.d. for 3 days) had no effect on the relaxation response to GTN in vitro (Fig. 1). Induction of GTN tolerance in vivo using transdermal GTN patches was evidenced by an inhibition of GTN-induced relaxation of isolated aorta from GTN-treated animals. However, coadministration of ZD2574 (100 mg kg^{-1} t.i.d. for 3 days) had no effect on GTN-induced relaxation in isolated aorta from tolerant animals (Fig. 2). The EC_{50} value for relaxation in control tissues was 1.6 ± 0.9 nM and was increased 5.3 ± 1.2-fold to 8.3 ± 1.6 nM in GTN-tolerant tissues. Treatment with ZD2574 did not significantly alter the EC_{50} value for relaxation in tolerant (17 ± 9.0 nM) tissues.

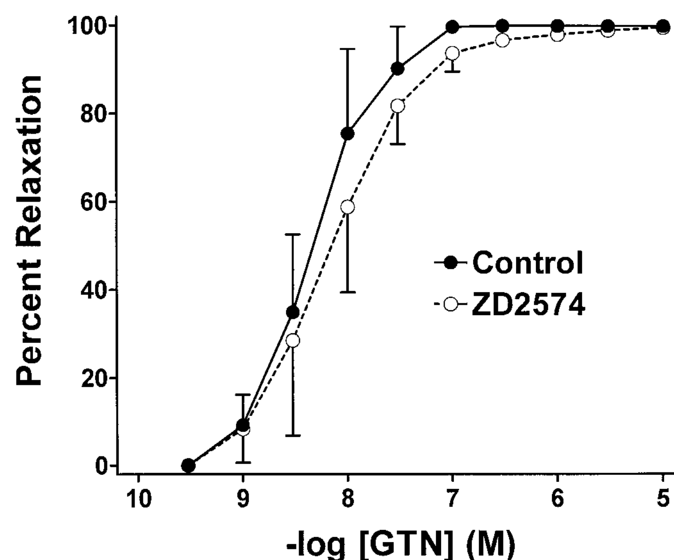

Fig. 1. Effect of ZD2574 (100 mg kg^{-1} i.p., 3 days) pretreatment on GTN-induced relaxation of isolated rat aorta. ZD2574 or vehicle was administered every 8 h by i.p. injection for 3 days. Data represent mean ± S.D. (n = 4).

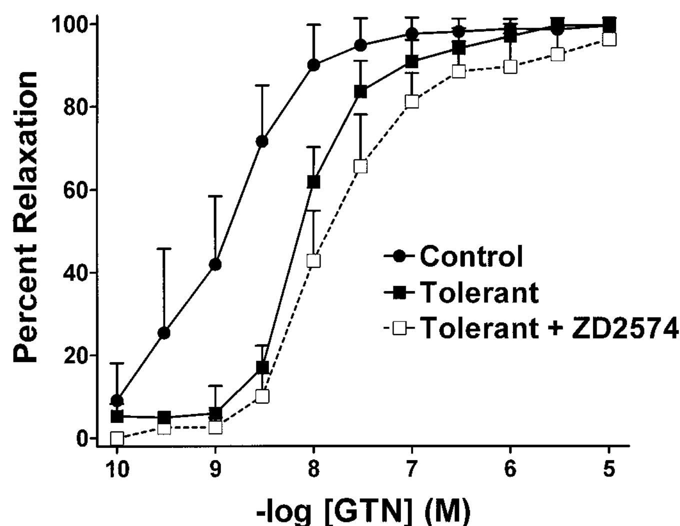

Fig. 2. Effect of tolerance and ZD2574 (100 mg kg^{-1} i.p.) on GTN-induced relaxation of isolated rat aorta. Control indicates rats not exposed to GTN and tolerant indicates rats given 0.4 mg h^{-1} GTN for 2 days. ZD2574 or vehicle was administered every 8 h by i.p. injection for 3 days. Data represent mean ± S.D. (n = 4–6).

Tissue Biotransformation Studies. Biotransformation of GTN was assessed in aorta removed from control or GTN-tolerant animals that had been treated with ZD2574 (100 mg kg^{-1} t.i.d. for 3 days) or drug vehicle (Fig. 3). In control tissues incubated with GTN, there was a selective formation of 1,2-GDN relative to 1,3-GDN formation, and the ratio of 1,2-GDN:1,3-GDN formation was decreased after the induction of in vivo GTN tolerance. These data are consistent with the alteration of regioselective biotransformation of GTN observed in blood vessels made tolerant to high doses of GTN in vitro (Brien et al., 1988; Slack et al., 1989). Administration of ZD2574 did not affect regioselective 1,2-GDN formation and coadministration of ZD2574 during the induction of in vivo tolerance did not alter the GTN tolerance-induced change in regioselective GDN formation.

cGMP Measurements. Similar to the biotransformation studies, GTN-induced cGMP accumulation was assessed in aorta from control or GTN-tolerant animals by exposing tissues to 2 μM GTN for 1 min (Fig. 4). With respect to basal cGMP levels, these were unaltered in aorta from GTN-tolerant animals or from animals treated with ZD2574 (100 mg kg^{-1} t.i.d. for 3 days). All groups demonstrated a significant increase in cGMP accumulation over basal levels after exposure to GTN. Differences between basal and GTN-induced cGMP accumulation were significantly larger in control groups (4.4 ± 0.91-fold and 3.5 ± 0.77-fold for vehicle and ZD2574, respectively) than for tolerant groups (1.8 ± 0.36-fold and 2.0 ± 0.28-fold for vehicle and ZD2574, respectively). There was a significant decrease in cGMP accumulation in GTN-tolerant tissues compared with control tissues. Consistent with the relaxation data, ZD2574 did not alter GTN-induced cGMP accumulation in control or GTN-tolerant animals.

Effect of ZD2574 on ET-1-Mediated Contraction of Rat Aorta In Vitro. Preincubation of aorta from control and GTN-tolerant rats with 500 nM ZD2574 resulted in an almost complete inhibition of the contractile response to ET-1 (Fig. 5), indicating that ZD2574 was able to effectively block the effects of ET-1 in rat aortic tissue in vitro. In contrast, the in vitro contractile response to ET-1 in tissues from GTN-tolerant animals or from tolerant animals coadministered ZD2574 in vivo was unaltered with respect to both the EC_{50} value for ET-1 (control, 4.2 ± 2.0 nM; tolerant, 4.3 ± 1.7 nM; tolerant plus ZD2574, 3.0 ± 1.7 nM) and the maximal contractile response (control, 0.94 ± 0.12 g; tolerant, 1.00 ± 0.14 g; tolerant plus ZD2574, 1.03 ± 0.05 g), indicating wash-

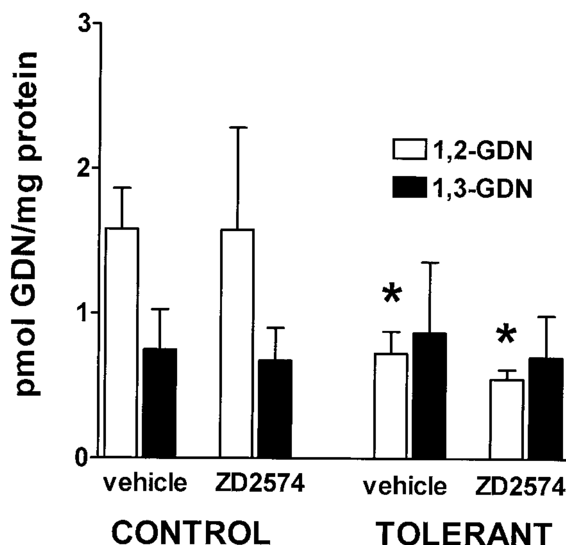

Fig. 3. Effect of tolerance and ZD2574 (100 mg kg^{-1} i.p.) on GTN biotransformation to 1,2-GDN and 1,3-GDN by isolated rat aorta. Control indicates rats not exposed to GTN and tolerant indicates rats given 0.4 mg h^{-1} GTN for 2 days. ZD2574 or vehicle was administered every 8 h by i.p. injection for 3 days. *P < .05 for formation of 1,2-GDN between control and tolerant groups. Data represent mean ± S.D. (n = 6).

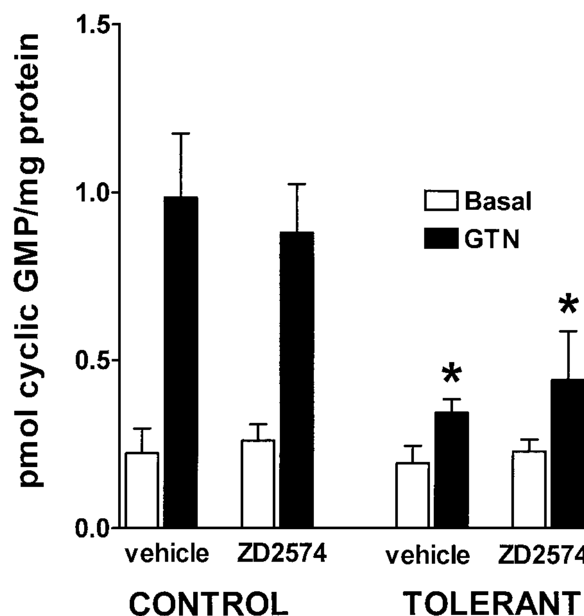

Fig. 4. Effect of tolerance and ZD2574 (100 mg kg^{-1} i.p.) on GTN-stimulated accumulation of cGMP in isolated rat aorta. Control indicates rats not exposed to GTN and tolerant indicates rats given 0.4 mg h^{-1} GTN for 2 days. ZD2574 or vehicle was administered every 8 h by i.p. injection for 3 days. Basal indicates nonstimulated cGMP levels and GTN indicates stimulation with 2 μM GTN for 1 min. *P < .05 for GTN-stimulated cGMP accumulation between control and tolerant groups. Data represent mean ± S.D. (n = 4).

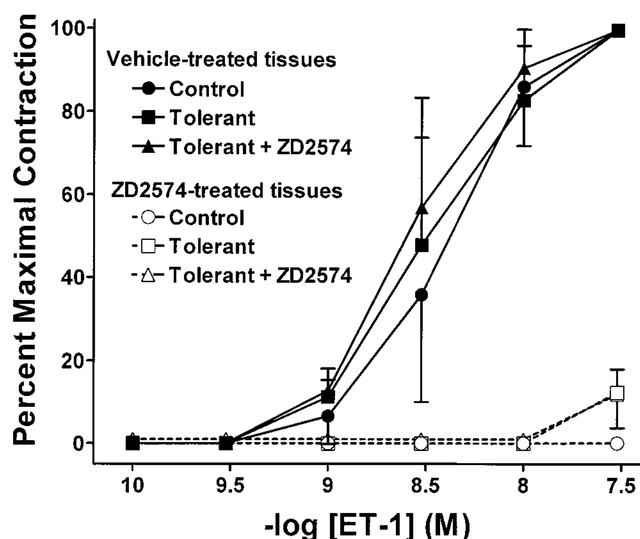

Fig. 5. Response to ET-1 in isolated rat aorta pretreated with 500 nM ZD2574 in vitro. Control indicates rats not exposed to GTN and tolerant indicates rats given 0.4 mg h^{-1} GTN for 2 days. Vehicle indicates tissue segments treated with drug vehicle and ZD2574 indicates tissue segments treated with 500 nM ZD2574 for 15 min. Data represent mean ± S.D. (n = 3).

out of the antagonist occurred during the preparation of the tissue. In a previous study it was reported that ET-1-mediated constriction of GTN-tolerant rabbit aorta was decreased and it was suggested that this was due to the binding of existing ET receptors by increased amounts of locally produced ET-1, thus rendering them unavailable for interaction with exogenously applied ET-1 (Münzel et al., 1995a). In our rat model of GTN tolerance, however, the contractile response to exogenous ET-1 in tolerant aortae was unaltered with respect to both the maximal contractile response and the EC_{50} value for ET-1. This suggests that to the extent that ET-1 is increased by GTN treatment, the contractile response to exogenous ET-1 is independent of endogenous ET-1 levels. These data also indicate that the in vitro responsiveness to ET-1 is unaltered during tolerance development and during chronic ET receptor blockade.

Effect of ZD2574 on ET-1-Mediated Changes in Blood Pressure. The change in MAP as assessed by measuring the AUC after an infusion of 2500 ng kg^{-1} ET-1 was significantly blunted for at least 8 h after 24-h treatment with ZD2574 (100 mg kg^{-1}) (Fig. 6). The baseline MAP for ZD2574-treated rats (100.2 ± 2.8 mm Hg) was not significantly different from that measured in control animals (105.2 ± 5.5 mm Hg). In addition, ET-1 did not cause a transient, initial decrease in MAP in ZD2574-treated animals, whereas this was observed in control (vehicle-treated) animals (Fig. 7). The vasodilator response to ET-1 has been attributed to endothelial NO release mediated by endothelial ET_B receptors, and thus it would appear that this dosage regimen of ZD2574 results in the blockade of both ET receptor subtypes. After this brief vasodilator response, ET-1 caused a rapid, acute increase in blood pressure in both control and ZD2574-treated animals, which returned to baseline in ZD2574-treated animals within 15 min after the ET-1 infusion, but remained elevated for 60 to 80 min in the control animals. It would appear that an exogenous, bolus infusion of ET-1 had an acute vasoconstrictor effect that was not blocked by ZD2574, whereas the later phase of the increase in MAP mediated by ET-1 was completely blocked by this compound.

Tissue Levels of ET-1. Tissue levels of ET-1 in control and tolerant aorta were measured by radioimmunoassay (Fig. 8) to determine whether the GTN tolerance induction protocol caused an increase in ET-1 levels as previously reported using immunocytochemical analysis (Münzel et al., 1995a). We also assessed ET-1 levels in another vascular tissue viz. vena cava. Total tissue levels of ET-1 were increased in tolerant rat aorta (720 ± 220 pg of ET-1 g of $tissue^{-1}$) by 2.3 ± 0.4-fold over levels measured in control aorta (310 ± 36 pg of ET-1 g of $tissue^{-1}$). Similarly, ET-1 levels were increased in tolerant rat vena cava (370 ± 110 pg of ET-1 g of $tissue^{-1}$) by 2.2 ± 0.5-fold over levels measured in control vena cava (170 ± 10 pg of ET-1 g of $tissue^{-1}$).

Discussion

The major finding in this study was that chronic antagonism of ET receptors by ZD2574 did not alter the tolerance-inducing effects of chronic GTN administration, despite the significant increase in tissue levels of ET-1 that were measured in rat blood vessels after the induction of GTN tolerance. These data would suggest that the increase in ET-1 is a consequence rather than a cause of GTN tolerance. The development of GTN tolerance in our in vivo rat model was characterized by a parallel, rightward shift in the concentration-response curve for GTN and an increased EC_{50} value for relaxation of aorta removed from GTN-tolerant animals (Fig. 2). To determine whether this correlated with biochemical changes, we assessed GTN biotransformation and GTN-induced cGMP accumulation. In GTN-tolerant rat aorta, there was a significant decrease in the vascular biotransformation of GTN (Fig. 3) to its 1,2-GDN metabolite, whereas 1,3-GDN levels remained unchanged. In nontolerant tissues, there is preferential formation of 1,2-GDN over 1,3-GDN (Brien et al., 1988; Bennett et al., 1994; McGuire et al., 1994). However, this regioselective biotransformation of GTN is reduced in both in vitro and in vivo models of tolerance, and the ratio of 1,2-GDN to 1,3-GDN is closer to 1:1 (Bennett et al., 1989; Slack et al., 1989). Consistent with the decrease in biotransformation observed, the increase in cGMP after exposure to GTN was significantly impaired in GTN-tolerant aorta (Fig. 4). These data are in agreement with previous reports showing that in GTN-tolerant tissues, GTN-induced stimulation of guanylyl cyclase was markedly diminished and cGMP phosphodiesterase activity was increased, results both of which correlated with a decrease in cGMP accumulation (Axelsson and Andersson, 1983; Waldman et al., 1986; Bennett et al., 1988).

ZD2574 did not alter the relaxation response to GTN in control (Fig. 1) or GTN-tolerant (Fig. 2) rat aorta and had no effect on the decrease in biotransformation of GTN (Fig. 3) or on the decrease in GTN-induced cGMP accumulation (Fig. 4) that was observed in GTN tolerant tissues. Although submicromolar concentrations of ZD2574 were clearly effective in antagonizing the contractile effects of ET-1 in vitro (Fig. 5), it was important to confirm that the 100 mg kg^{-1} t.i.d. dosage regimen for ZD2574 was sufficient to block the in vivo effects of ET-1 at the ET_A receptor. The exogenous application of ET-1 in anesthetized rats causes an initial transient decrease in blood pressure followed by a sustained increase (Yanagi-

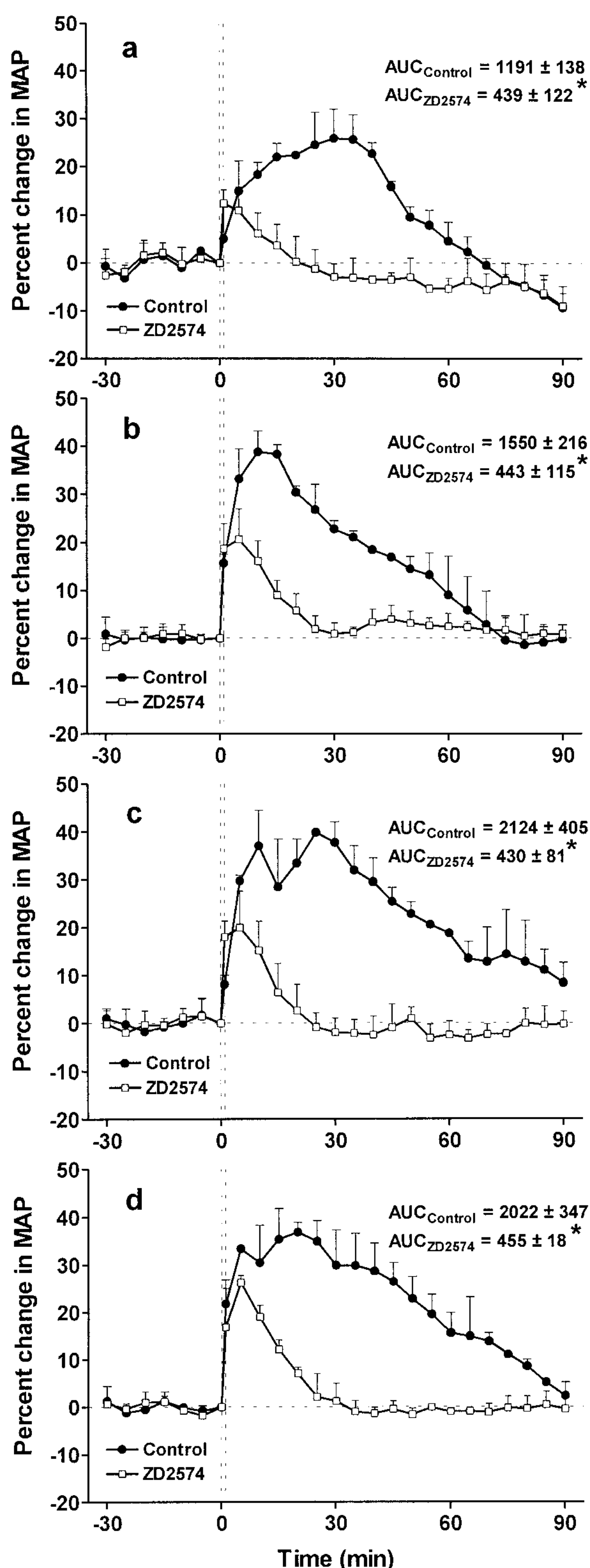

Fig. 6. Effect of ZD2574 (100 mg kg^{-1} i.p.) on the percentage increase in MAP after a bolus i.v. infusion of 2500 ng kg^{-1} ET-1 at 2 (a), 4 (b), 6 (c), and 8 h (d) after the last dose of ZD2574. The dotted lines define the 1-min period over which the ET-1 bolus was administered. For clarity, the initial transient decrease in MAP during the administration of ET-1 has been omitted (see Fig. 7). Control indicates rats administered drug vehicle and ZD2574 indicates rats administered ZD2574 by i.p. injection every 8 h for 24 h (four doses in total). $^{*}P < .05$ for the AUC for ZD2574-treated animals compared with control (Student's t test for unpaired data). Data represent mean ± S.D. (n = 3).

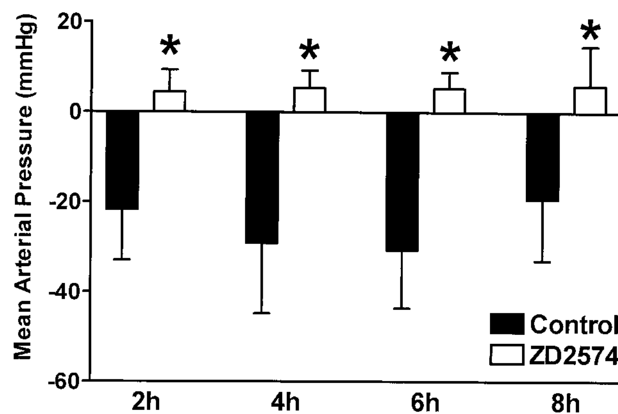

Fig. 7. Effect of ZD2574 (100 mg kg^{-1} i.p.) on the change in MAP during the bolus i.v. infusion of 2500 ng kg^{-1} ET-1 at 2, 4, 6, and 8 h after the last dose of ZD2574. Control indicates rats administered drug vehicle and ZD2574 indicates rats administered ZD2574 by i.p. injection every 8 h for 24 h (four doses in total). $^{*}P < .05$ for the change in MAP for ZD2574-treated animals compared with control (Student's t test for unpaired data). Data represent mean ± S.D. (n = 3).

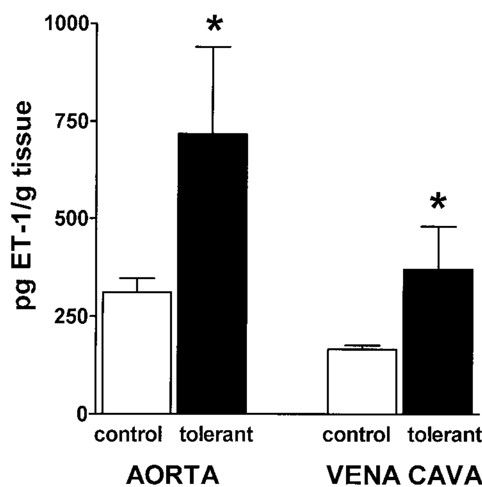

Fig. 8. Effect of tolerance on the rat aortic and pooled vena caval tissue levels of ET-1 measured by radioimmunoassay. Control indicates rats not exposed to GTN and tolerant indicates rats given 0.4 mg h^{-1} GTN for 2 days. $^{*}P < .05$ for the increase in levels of ET-1 in tolerant animals compared with control (Student's t test for unpaired data). Data represent mean ± S.D. (n = 5, aorta; n = 4, vena cava).

sawa et al., 1988). The data in Fig. 6 indicate that a 24-h pretreatment with ZD2574 was sufficient to block the sustained increase in MAP caused by a bolus i.v. infusion of ET-1, and that this inhibitory effect was maintained for at least 8 h after the last dose of ZD2574. There was, however, an initial increase in MAP that was not blocked by ZD257. A reasonable explanation that could account for this is that after a bolus infusion of ET-1, the vasculature would be initially exposed to a high concentration of ET-1. Because of the competitive nature of the ET_A-selective antagonist used in our studies, this initial high dose of ET-1 may have been sufficient to displace the antagonist from the receptor, allowing ET-1 to bind and cause an acute vasoconstrictor response. However, as ET-1 distributes and the concentration throughout the vasculature decreases, ZD2574 would effectively compete with circulating ET-1 for the ET_A receptor and the MAP would quickly return to its pre-ET-1 infusion level. It was our assumption that during the development of GTN tolerance,

endogenous ET-1 levels would be expected to increase slowly so that the competitive blockade of ET_A receptors by the large dose of ZD2574 used would be sufficient to block the effects of any increase in ET-1 levels.

In in vitro receptor binding studies, ZD2574 exhibits about a 500-fold selectivity for ET_A receptors (R. A. Bialecki, Zeneca Pharmaceuticals, personal communication). However, with the high doses of ZD2574 used in the current study, in addition to the inhibition of ET-1 mediated vasoconstriction, ZD2574 abolished the initial transient depressor response caused by ET-1 (Fig. 7). This indicated that ZD2574 had lost its selectivity for ET_A receptors under these in vivo conditions, and was also having an inhibitory effect at endothelial ET_B receptors. Thus, the inhibitory effect of ZD2574 on the depressor and pressor effects of ET-1 is similar to that of the nonselective ET receptor antagonist bosentan, which inhibits both the initial transient depressor response after ET-1 administration as well as the prolonged pressor response (Clozel et al., 1994).

Münzel et al. (1995a) first suggested a role for ET-1 in GTN tolerance and showed that isolated aorta from rabbits made tolerant to GTN in vivo exhibited a hypersensitive in vitro contractile response to the vasoconstrictors Ang II, serotonin, phenylephrine, potassium chloride, and phorbol-12,13-dibutyrate, an effect that was reversed in vitro by the PKC inhibitors calphostin C and staurosporine. Consistent with these results, the sensitivity for phenylephrine-induced contraction was increased in aortae from GTN-tolerant rats (this study, data not shown; De la Lande et al., 1999). In a more recent study, the PKC inhibitors chelerythrine and Gö 6976 were found to partially restore the vasodilator response to GTN in aortae from GTN-tolerant rats (Münzel et al., 2000). In a third study, it was reported that the in vivo coadministration of GTN and the PKC inhibitor *N*-benzoyl-staurosporine blocked the development of tolerance, inasmuch as the relaxation responses to GTN in aortae from control and GTN-tolerant, *N*-benzoyl-staurosporine-treated rats were not different. Although the prevention of GTN tolerance by coadministration of a PKC inhibitor (Zierhut and Ball, 1996) would be consistent with the proposal that PKC activity is increased during the development of GTN tolerance, an alternative explanation is that basal PKC activity acts to physiologically antagonize the actions of GTN. Because the net effect of GTN action is to decrease the levels of intracellular Ca^{2+} or to desensitize the contractile apparatus to Ca^{2+}, inhibition of PKC may simply increase the sensitivity of vascular smooth muscle to the vasodilator effects of GTN and not actually prevent the development of tolerance.

Because it has been proposed that PKC activation during the development of GTN tolerance could be due to an increase in ET-1 production and increased levels of ET-1 receptor activation (Münzel et al., 1995a), inhibition of the ET_A receptor by ZD2574 might be expected to block the activation of PKC by ET-1, thereby preventing or minimizing the development of tolerance. The results of the current study indicate that chronic ET_A receptor antagonism did not prevent the development of tolerance to GTN. However, levels of PKC activity were not measured in this study, or in any other study examining GTN tolerance, and may have been elevated due to the influence of other vasoactive substances, not ET-1 acting at the ET_A receptor. There is evidence to suggest that other vasoactive substances such as Ang II are elevated during the development of GTN tolerance (Parker and Parker, 1992; Kurz et al., 1999) and in addition to ET-1, many other endogenous substances (Ang II, serotonin, and norepinephrine) are known to activate PKC (Lee and Severson, 1994).

The results of the current study contrast with those of a recent study by Kurz et al. (1999), in which coadministration of the nonselective ET receptor antagonist bosentan during in vivo tolerance induction in rabbits with GTN patches resulted in a partial restoration of the relaxation response in aorta from these animals, suggesting that there is a role for ET-1 in reducing the vasodilator response to GTN in tolerance. The reasons for this discrepancy are unclear because the protocols for tolerance induction and treatment with ET receptor antagonists were similar. As mentioned above, the dosage regimen used for ZD2574 resulted in complete inhibition of the initial transient vasodilator response to ET-1, indicating that endothelial ET_B receptors were also blocked by the antagonist. Thus, it is unlikely that the difference in results between the two studies is due to differential effects on ET receptor subtypes. It may be that the mechanisms for tolerance development differ between the two species. In this regard, Münzel et al. (1995b) found that the vasodilator response to GTN in tolerant rabbit aorta was increased upon removal of the endothelium, and that there was a substantial reduction in the vasodilator response to acetylcholine in GTN-tolerant aortae. In contrast, the vasodilator response to GTN in GTN-tolerant rat aorta is unaltered after removal of the endothelium, and there is very little or no cross-tolerance to acetylcholine (De la Lande et al., 1999; Ratz et al., 2000).

Many endogenous substances and physical stimuli have been reported to alter the production and release of ET-1, including Ang II and vasopressin (Emori et al., 1991), as well as hypoxia (Kourembanas et al., 1991) and shear stress (Kuchan and Frangos, 1993). There is evidence that NO is involved in the regulation of ET-1 production and release (Boulanger and Lüscher, 1990; Kourembanas et al., 1993), and that NO plays a role in the termination of ET-1 signaling either by directly displacing ET-1 from its receptor or by interfering with the pathway for Ca^{2+} mobilization (Goligorsky et al., 1994). One could speculate that as an adaptive response to the increased vascular levels of NO from the biotransformation of GTN, there would be an initial decrease in ET-1 production and release. However, as GTN tolerance develops and the vascular biotransformation of GTN to NO is abolished, this component of NO-dependent regulation of ET-1 would be absent, and a rebound increase in ET-1 levels could occur. In this scenario, the observed increase in tissue levels of ET-1 would be a consequence of chronic GTN exposure, rather than a cause of GTN tolerance.

The results of this study that show an increase in tissue levels of ET-1 after the development of GTN tolerance may have implications regarding the clinical treatment of congestive heart failure with GTN. A large volume of evidence has been accumulating to suggest that ET-1 levels are increased in congestive heart failure (Love and McMurray, 1996) and may contribute to the increase in vascular tone inherent in this pathophysiological condition (Teerlink et al., 1994). It has also been shown that the levels of ET-1 correlate directly with the severity of the disease and are a major predictor of morbidity (Mulder et al., 1997). Because GTN is commonly used in the treatment of congestive heart failure, it would be

beneficial to develop a more complete understanding of the effects that long-term GTN treatment have on the levels of ET-1. Perhaps intermittent dosing regimens are sufficient to prevent GTN-mediated increases in ET-1. However, the effects of short-term GTN exposure should also be investigated to determine whether they cause an increase in ET-1 levels. In this case, the benefits of GTN treatment in congestive heart failure may be offset by a GTN-mediated increase in ET-1 levels. This would provide a rationale for the coadministration of an ET-1 receptor antagonist during GTN therapy.

In conclusion, the evidence presented in this study indicates that chronic inhibition of ET_A receptors, and also ET_B receptors, by ZD2574 was not sufficient to prevent or diminish the tolerance-inducing effects of GTN in the rat. In the absence of an effect of ET_A/ET_B receptor antagonism, the increase in ET-1 levels observed after the induction of GTN tolerance does not suggest a primary role for ET-1 in mediating GTN tolerance, but rather that elevated ET-1 levels may occur as a consequence of the vascular changes that occur during chronic GTN exposure.

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