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Endothelial nitric oxide synthase is a site of superoxide synthesis in endothelial cells treated with glyceryl trinitrate

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1 Tolerance to glyceryl trinitrate (GTN) involves superoxide ($O_2^{\cdot-}$) production by endothelial cells. Nitric oxide synthase (NOS) produces $O_2^{\cdot-}$ when L-arginine (L-arg) is limited. The purpose of this study was to test the hypothesis that GTN stimulates NOS to increase $O_2^{\cdot-}$ synthesis in endothelial cells when L-arg is limited.

2 Production of $O_2^{\cdot-}$ by bovine aortic endothelial cells (BAEC, passages 3–5) was determined by spectrophotometrically measuring superoxide dismutase-inhibited reduction of ferricytochrome C to ferrocytochrome C. Cells were incubated in buffer without L-arg. $O_2^{\cdot-}$ production was measured using BAEC either untreated or treated with L-NAME or L-arg alone or following treatment with GTN (10^{-9} to 10^{-6} M) for 30 min or DPTA NONOate (10^{-7} and 10^{-6} M) alone or with GTN or DPTA NONOate after pretreatment with nitro-L-arginine methyl ester (L-NAME), L-arg or their inactive enantiomers, D-NAME or D-arg (all 5×10^{-4} M) ($n = 6-7$/group).

3 L-NAME alone produced a 69% reduction in $O_2^{\cdot-}$ levels. Treatment with L-arg alone had no effect. Cells treated with GTN alone exhibited an increase in $O_2^{\cdot-}$. This effect was prevented by pretreatment with either L-NAME or L-arg, and was unaffected by D-NAME or D-arg. We observed a dose-response relationship in $O_2^{\cdot-}$ production to GTN over a range of 10^{-9} to 10^{-7} M.

4 The NO donor, DPTA-NONOate, unlike GTN, did not have a significant effect on $O_2^{\cdot-}$ production.

5 In conclusion, endothelial NOS is a site of $O_2^{\cdot-}$ synthesis in endothelial cells activated by GTN. *British Journal of Pharmacology* (2000) **131,** 1019–1023

Keywords: Glyceryl trinitrate (nitroglycerin); endothelial nitric oxide synthase; L-arginine; nitrate tolerance; superoxide anion

Abbreviations: BAEC, bovine aortic endothelial cells; D-arg, D-arginine; D-NAME, nitro-D-arginine methyl ester; DPTA NONOate, dipropylenetriamine NONOate; GTN, nitroglycerin (glyceryltrinitrate); L-arg, L-arginine; L-NAME, nitro L-arginine methylester; L-NMMA, NG monomethyl L-arginine; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NOS, nitric oxide synthase; $O_2^{\cdot-}$, superoxide anion

Introduction

Previous studies have shown $O_2^{\cdot-}$ production by endothelial cells treated with a Ca^{2+}-ionophore or bradykinin (Katusic & Vanhoutte, 1989; Holland *et al*., 1990) and also by GTN, a NO donor (Dikalov *et al*., 1998). In general, sites of $O_2^{\cdot-}$ synthesis include membrane-bound NAD(P)H oxidases (Münzel *et al*., 1995), xanthine oxidase (Katusic & Vanhoutte, 1989), mitochondrial electron transport chain (Jansson *et al*., 1993), cytochrome P450 oxidase (Jansson *et al*., 1993) and cyclo-oxygenase (Holland *et al*., 1990). In a recent study of rat aortic endothelial cells (Puey *et al*., 1998), $O_2^{\cdot-}$ production in response to angiotensin II was blocked by pretreatment with L-NMMA, an inhibitor of eNOS, indicating that eNOS is yet another site of $O_2^{\cdot-}$ production. When the supply of L-arginine to eNOS is limited, eNOS utilizes oxygen as its principal substrate and produces $O_2^{\cdot-}$ (Pritchard *et al*., 1995; Presta *et al*., 1997; Ogonowski *et al*., 2000). In this study we test and confirm the hypothesis that eNOS is a site of $O_2^{\cdot-}$ synthesis in endothelial cells activated by GTN when L-arginine is limited. The results of this study amplify our recent findings that agonists of eNOS increase superoxide production when L-arginine is restricted (Ogonowski *et al*., 2000) and that L-arginine is depleted by GTN, indicating a component of nitroglycerin tolerance that is L-arginine dependent (Abou-Mohamed *et al*., 2000).

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Methods

Drugs and chemicals

Preservative free glyceryl trinitrate (Perlinganit®) was a kind gift of Dr Andreas Reimann of the Schwarz Pharma Company (Monheim, Germany). All other chemical reagents were purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.).

Cellular superoxide anion formation in the presence of glyceryl trinitrate

Primary cultures of bovine aortic endothelial cells (BAEC) were prepared from fresh blood vessels as described previously (Behzadian *et al*., 1995). The production of $O_2^{\cdot-}$ by BAEC was determined by spectrophotometrically measuring the superoxide dismutase-inhibitable reduction of ferricytochrome C according to Pritchard *et al*., (1995). BAEC (passages 3–5) were plated in 35 mm dishes containing 10.5×20 mm fibronectin-coated *Thermanox* coverslips. After

reaching confluency in a M-199 medium containing 5×10^{-5} M L-arg, cells were washed three times (3 ml) with Dulbecco's phosphate buffered saline (DPBS) and two coverslips were placed in a disposable plastic cuvette facing each other. DPBS (1.8 ml) without L-arg was gently placed in a cuvette in addition to 200 μl of ferricytochrome C (final concentration, 5×10^{-6} M). The cuvette was inverted to mix the reagents. This was followed by a 30 min incubation period at 37°C to provide for the cells to equilibrate. After this period, the test substance or an equal volume of buffer (10 μl) was added and mixed. Absorbance was then recorded for 60 min using a spectrophotometer at 550 nm wavelength to monitor basal $O_2^{\cdot -}$ production or the effect of pretreatments. Production of $O_2^{\cdot -}$ was evident within minutes. The effects of pretreatment with L-NAME or L-arginine (L-arg, 5×10^{-4} M) on basal $O_2^{\cdot -}$ production were also determined. In order to determine whether addition of the L-arg analogue L-NAME or L-arg could prevent or reduce the formation of superoxide anion, experiments were performed in which L-NAME (5×10^{-4} M) or L-arg (5×10^{-4} M) were added prior to GTN treatment. Change in absorbance over time was recorded in the presence or absence of superoxide dismutase (400 u ml) to determine the portion of absorbance change due to $O_2^{\cdot -}$ production. The amount of superoxide anion $[\varepsilon = 2100\ \text{cm}^{-1})/(\text{mol l})^{-1}]$ generated was determined over time and reported as pmoles $O_2^{\cdot -}$ min^{-1} $(10^6\ \text{cells})^{-1}$. In order to evaluate the possible scavenging action of L-NAME and L-arg (Rehman *et al*., 1997), pretreatments of D-arg or D-NAME (both 5×10^{-4} m) were used as controls. In other experiments, varied concentrations of GTN (10^{-9} to 10^{-6} M) were added to the cuvette after the initial incubation period. We also examined the effects of the NO donor, DPTA-NONOate (at 10^{-7} and 10^{-6} M) on $O_2^{\cdot -}$ production.

Statistical analysis

Data are presented as mean ± s.e.mean of the indicated number of observations (n) and the difference between groups was assessed using the paired t-test or analysis of variance, when appropriate. A probability value (P) less than 0.05 was considered to be statistically significant.

Results

Basal production of $O_2^{\cdot -}$ by BAEC in L-arg free media was 59 pmol min^{-1} $(10^6\ \text{cells})^{-1}$ (Figure 1). Pretreatment of control cells with L-NAME suppressed basal production of $O_2^{\cdot -}$ by 70%. Pretreatment of these cells with L-arg did not alter basal $O_2^{\cdot -}$ production.

Treating cells with GTN (10^{-6} M) increased $O_2^{\cdot -}$ production from 69 to 155 pmol min^{-1} $(10^6\ \text{cells})^{-1}$, an increase of 125% over basal levels (Figure 2A). Experiments performed in parallel showed that treatment with L-NAME prior to GTN decreased $O_2^{\cdot -}$ production to 15 pmol min^{-1} $(10^6\ \text{cells})^{-1}$, a decrease of 140 pmol min^{-1} $(10^6\ \text{cells})^{-1}$, i.e. 90% of levels for GTN alone or 79% of control levels (Figure 2B vs 2A). Pretreatment of cells with L-arg also blocked the effect of GTN in increasing $O_2^{\cdot -}$ production (Figure 2B). L-arg pretreatment reduced $O_2^{\cdot -}$ levels to 88 pmol min^{-1} $(10^6\ \text{cells})^{-1}$. This value is 43% of the response to GTN alone and indicates that L-arginine pretreatment reduced the $O_2^{\cdot -}$ response to treatment with GTN to basal (cells alone) levels (compare Figure 2A,B)].

In contrast, treating cells with either D-NAME or D-arg prior to the GTN treatment did not significantly change the responses to GTN which were increased by 150 and 86 per cent, respectively, over basal control (compare Figure 2A,C). Cells receiving pretreatments with D-NAME or D-arg and then treated with GTN were observed to have increases in $O_2^{\cdot -}$ production of 175 pmol min^{-1} $(10^6\ \text{cells})^{-1}$ for D-NAME and 130 pmol min^{-1} $(10^6\ \text{cells})^{-1}$ for D-arg (Figure 2C). Neither of these responses were different from cells treated with only GTN (compare Figure 2A,C); however, there is a difference between the effects of D-arg and L-arg (compare Figure 2B,C). All experiments displayed in Figure 2 were performed in parallel.

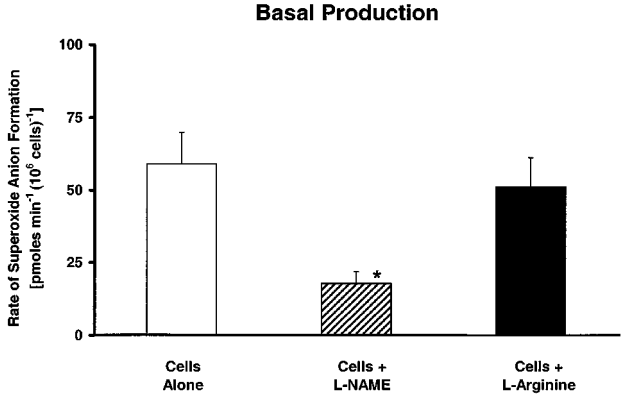

Figure 1 Basal production of $O_2^{\cdot -}$ by BAEC in L-arg free media. Effects of pretreatment of cells with L-NAME (5×10^{-4} M) or L-arginine (5×10^{-4} M) are also displayed. *Indicates difference from basal production of $O_2^{\cdot -}$ ($P < 0.01$). n for each experiment ranged from 6–7.

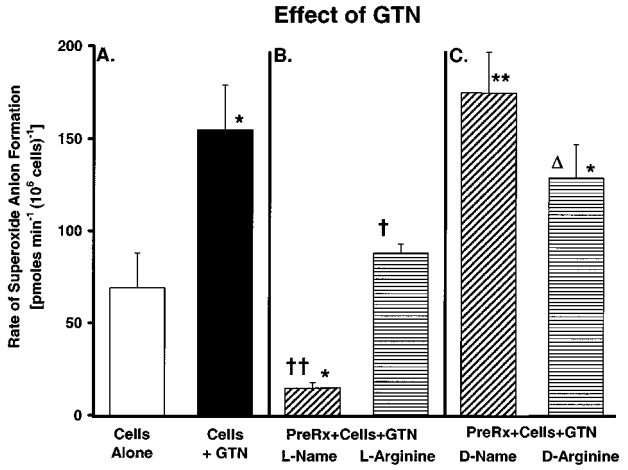

Figure 2 Effect of GTN (10^{-6} M) on $O_2^{\cdot -}$ production by endothelial cells. (A) shows basal production of $O_2^{\cdot -}$ in the absence of L-arginine and the effect of treatment with GTN (10^{-6} M). (B) shows the effect on $O_2^{\cdot -}$ production of pretreating cells with L-NAME or L-arg followed by treatment with GTN. (C) shows the effect on $O_2^{\cdot -}$ production of pretreating cells with D-NAME or D-arginine followed by treatment with GTN. *Indicates difference from basal $O_2^{\cdot -}$ production, $P < 0.05$, **indicates $P < 0.005$, †indicates difference from cells treated with GTN alone, $P < 0.05$, ‡indicates $P < 0.01$. In A, there is a 125% increase in $O_2^{\cdot -}$ production when cells (in L-arginine free buffer) are treated with GTN alone. In B L-NAME pretreatments resulted in a decrease in $O_2^{\cdot -}$ which is different from both basal and GTN stimulated $O_2^{\cdot -}$ production, whereas L-arginine pretreatment is different from GTN stimulated $O_2^{\cdot -}$ production only. In C, both D-NAME and D-arginine pretreatments resulted in increased $O_2^{\cdot -}$ production over basal production; neither treatment resulted in changes in $O_2^{\cdot -}$ production which were different from cells treated with GTN alone. Δ Indicates difference from cells treated with L-arginine, $P < 0.05$. n for each experiment ranged from 6–7.

In another set of parallel experiments, we compared responses to GTN at concentrations from 10^{-9} M, to 10^{-6} M (Figure 3). This study revealed that treatment with 10^{-9} M GTN did not alter levels of $O_2^{\cdot-}$. With 10^{-8} and 10^{-7} M GTN, $O_2^{\cdot-}$ production was significantly increased from 67 to 138 and 172 pmol min^{-1} $(10^6$ cells$)^{-1}$), respectively. With 10^{-6} M GTN, $O_2^{\cdot-}$ production was elevated over basal levels (131 pmol min^{-1} $(10^{-6}$ cells$)^{-1}$) but was significantly lower than observed with the 10^{-7} M concentration of GTN. These elevations in $O_2^{\cdot-}$ production by GTN were also inhibited by prior treatment with either L-NAME or L-arg (Table 1).

The NO donor, DPTA–NONOate did not have a significant action on superoxide production (Table 2). In comparison to GTN, addition of DPTA-NONOate resulted in $O_2^{\cdot-}$ production of 64 and 66 pmol min^{-1} $(10^6$ cells$)^{-1}$ at 10^{-7} and 10^{-6} M, respectively, compared to a basal level of 59 pmol min^{-1} $(10^6$ cells$)^{-1}$ for cells alone.

Discussion

Our data confirm the previous studies that endothelial cell NOS is a site of $O_2^{\cdot-}$ synthesis (Huk *et al*., 1997; Münzel *et al*., 2000; Ogonowski *et al*., 2000; Puey *et al*., 1998). These data also demonstrate that GTN can stimulate NOS-mediated production of $O_2^{\cdot-}$ in endothelial cells when L-arginine is limited. This conclusion is supported by the reduced $O_2^{\cdot-}$ production to GTN in cells pretreated with L-NAME or L-arginine. In contrast, neither D-NAME nor D-arginine pretreatments prevented the GTN induced rise in $O_2^{\cdot-}$ production and neither yielded results different from cells treated with GTN alone. This finding, along with the finding of a difference between treatments with D- and L-arg, excludes a previously observed scavenging action of D-NAME and L-NAME and D-arginine and L-arginine on $O_2^{\cdot-}$ (Rehmann *et al*., 1997) as an explanation for the findings of our study.

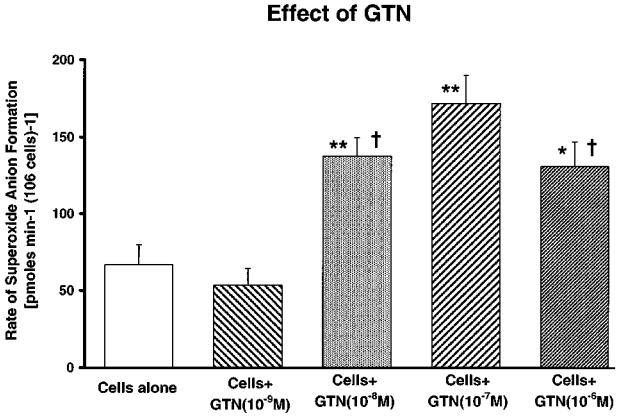

Figure 3 Comparison of the effects of GTN at concentrations from 10^{-9} to 10^{-6} M on production of $O_2^{\cdot-}$. *Indicates difference from basal $O_2^{\cdot-}$ production, $P<0.01$, **indicates $P<0.005$, †indicates difference from cells treated with GTN 10^{-7} M, $P<0.05$. *n* for each experiment ranged from 6–7.

Whereas L-arginine pretreatment of cells prior to GTN treatment resulted in $O_2^{\cdot-}$ values unchanged from control (cells alone), L-NAME pretreatment caused a significant decrease from control. Our present and previous data suggest that L-NAME inhibits both basal and stimulated $O_2^{\cdot-}$ production, while L-arginine inhibits only stimulated $O_2^{\cdot-}$ production (Ogonowski *et al*., 2000). We believe this difference is due to direct inhibition of eNOS activities in producing both NO and $O_2^{\cdot-}$ by L-NAME pretreatment. By contrast, we believe that addition of L-arginine supports NO production and diminishes $O_2^{\cdot-}$ production, i.e. shifts eNOS activity back into producing NO. Production of $O_2^{\cdot-}$, which remains after L-NAME pretreatment, is felt to be from sites other than eNOS. Similar observations on the effect of NOS inhibitors have been made by others (Huk *et al*., 1997; Münzel *et al*., 2000; Ogonowski *et al*., 2000; Puey *et al*., 1998). It is interesting to note that basal $O_2^{\cdot-}$ production in our system is much less than basal $O_2^{\cdot-}$ levels observed by others in human umbilical vein endothelial cells in arginine-free buffer (Prichard *et al*., 1995).

Collectively, these data indicate that $O_2^{\cdot-}$ synthesis by endothelial cells treated with GTN involves eNOS activation. The findings of this study are in agreement with those of an earlier study by our group (Abou-Mohamed *et al*., 2000) which indicated that in addition to GTN's well known actions as an NO donor, it has an auxiliary action involving activation of eNOS.

Our finding that treatment with a 10^{-7} M concentration of GTN resulted in a higher level of $O_2^{\cdot-}$ production than with the 10^{-6} M concentration is consistent with the known avid reactivity of $O_2^{\cdot-}$ with NO to form peroxynitrite ($ONOO^-$) (Huie & Padmaja, 1993; Amirmansour *et al*., 1999). Increasing concentrations of GTN will donate greater amounts of NO which will decrease levels of $O_2^{\cdot-}$ through the mechanism of $ONOO^-$ formation. Related interactions and a decrease in $O_2^{\cdot-}$ have been noted by others (Kooy & Royall, 1994; Amirmansour *et al*. 1999).

Control studies with the NO donor DPTA-NONOate demonstrated specificity of the GTN effect. We used a

Table 2 Effect of DPTA-NONOate (DPTA) on $O_2^{\cdot-}$ production in BAEC

Control [pmol min^{-1} $(10^6$ cells$)^{-1}$]	*DPTA* (10^{-7} M) [pmol min^{-1} $(10^6$ cells$)^{-1}$]	*DPTA* (10^{-6} M) [pmol min^{-1} $(10^6$ cells$)^{-1}$]
59±12	64±10	66±11

n for each experiment ranged from 6–7.

Table 1 Effect of pretreatment of BAEC with L-NAME (5×10^{-4} M) or L-arginine (5×10^{-4} M) on $O_2^{\cdot-}$ production in response to GTN

Concentration of GTN	*GTN alone*[a] [pmol min^{-1} $(10^6$ cells$)^{-1}$]	*GTN after L-NAME* [pmol min^- $(10^6$ cells$)^{-1}$]	*GTN after L-arginine* [pmol min^- $(10^6$ cells$)^{-1}$]
10^{-9} M	53±11	25±5*	51±10
10^{-8} M	138±12	28±6**	62±9**
10^{-7} M	172±17	22±4**	54±11**
10^{-6} M	131±16	29±5**	60±8*

[a]Compare to basal production of 67±11 units. *n* for each experiment ranged from 6–7. *Indicates difference from values for that concentration of GTN alone, $P<0.05$, **indicates $P<0.001$.

NONOate for these experiments in order to rule out the possible contribution of GTN's function as a NO donor in the $O_2^{\cdot-}$ response. Unlike the syndomomines, such as SIN-1, which are capable of generating $O_2^{\cdot-}$ as well as donating NO (Feelisch *et al*., 1989), NONOates have been shown to function as NO donors without generating $O_2^{\cdot-}$ (Wink *et al*., 1996; Rosenberg *et al*., 1999). In marked contrast with GTN, superoxide levels in the cells treated with NONOate at both 10^{-7} and 10^{-6} M were unchanged from basal values. We believe this indicates that the responses seen with GTN reflect its function as a NOS agonist function rather than as a NO donor.

It could be argued that the rate of release of NO from the two NO donors are different and at the concentrations used for DPTA-NONOate, little or no increase of NO may occur, resulting in no effect on NOS activity. However, 10^{-6} M DPTA-NONOate is chemically equivalent to 2×10^{-6} M of NO. DPTA-NONOate has a $T_{1/2}$ of 180 min (Mooradian *et al*., 1995) and release of NO follows first order kinetics (Maragos *et al*., 1991). Therefore, 10^{-6} M DPTA-NONOate at 60 min will yield $\sim0.33\times10^{-6}$ M NO. We have previously determined that 10^{-6} M DPTA-NONOate decreases system y^+ uptake of L-arginine in endothelial cells by about 22% at 60 min (Ogonowski *et al*., 2000). This observation strongly indicates that an adequate amount of NO is released at 1 h to affect cell function.

Although one might expect that NONOate would have reduced the measured $O_2^{\cdot-}$ levels due to formation of $ONOO^-$, this did not occur in our study. The lack of $O_2^{\cdot-}$ reduction in the DPTA-NONOate-treated cultures may be explained by the failure of NO to reach critical levels required for $ONOO^-$ formation (Amirmansour *et al*., 1999).

We did not directly identify the NOS isoform stimulated by GTN in our experiments. The predominant isoform in BAEC is eNOS or NOS type III, but iNOS or NOS type II may be induced by cytokine treatment (Kaku *et al*., 1997). Therefore, $O_2^{\cdot-}$ production from iNOS might occur in some experimental conditions. However, basal levels of NO production in our endothelial cells are in line with only normal eNOS activity (Abou-Mohamed *et al*., 2000). As shown by Kaku *et al*., (1997), 8–16 h are required for a measurable increase in NO production due to induction of iNOS expression. In our study, the increased $O_2^{\cdot-}$ production resulting from GTN treatment occurred rapidly, within only 30 min of GTN addition. Therefore, any contribution of iNOS to $O_2^{\cdot-}$ production seen in this study is considered unlikely.

Our findings of eNOS as a site of $O_2^{\cdot-}$ production in response to GTN are in general agreement with those of another recent study (Münzel *et al*., 2000). Additionally, our experiments limiting the supply of L-arginine demonstrate directly for the first time that GTN-stimulated $O_2^{\cdot-}$ production occurs as a result of reduced L-arginine availability to eNOS. It has previously been reported that $O_2^{\cdot-}$ production occurs only with *in vivo* models of nitrate tolerance (Münzel *et al*., 1995). Our data plus those of (Dikalov *et al*., 1998) suggest that $O_2^{\cdot-}$ production also occurs in *in vitro* models of GTN tolerance. Our observation that addition of extracellular L-arginine prevents GTN-induced $O_2^{\cdot-}$ formation suggests that the prime source of this substrate for endothelial NOS is the extracellular fluid.

We believe these data shed new light on the long-standing problem of nitrate tolerance. Nitrate tolerance has been regarded as a problem with multiple etiologies (Parker & Parker, 1998). Recently, the oxidative hypothesis (Münzel *et al*., 1995) has gained considerable attention. According to this hypothesis, $O_2^{\cdot-}$ production results from activation of membrane-bound AND(P)H oxidase by angiotensin II, which is enhanced by GTN. This leads to scavenging of NO by $O_2^{\cdot-}$ to form peroxynitrite and results in reduced NO for vasorelaxation, i.e. tolerance. Recent work by Milone *et al*., (1999) raises questions about this hypothesis. In that study, an angiotensin II receptor antagonist, losartan, was ineffective in preventing tolerance to GTN. On the other hand, supplemental L-arginine seems to be beneficial in patients with angina treated with nitrates (Ceremuzynski *et al*., 1997; Kaesemeyer *et al*., 1997). These patients exhibited increased treadmill exercise time and reversal of tolerance to GTN.

In conclusion, endothelial cell NOS is a site of $O_2^{\cdot-}$ production in endothelial cells treated with GTN. Both L-NAME and L-arginine, but not their inactive enantiomers, reduced $O_2^{\cdot-}$ synthesis. This indicates that NOS activation by GTN, not scavenging by L-NAME and L-arginine, is involved. These findings suggest that supplemental L-arginine may be effective in reducing tolerance to GTN.

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