Nitroglycerin metabolism in vascular tissue: role of glutathione S-transferases and relationship between NO[•] and NO₂⁻ formation

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Nitroglycerin is a commonly employed pharmacological agent which produces vasodilatation by release of nitric oxide (NO[•]). The mechanism by which nitroglycerin releases NO[•] remains undefined. Recently, glutathione S-transferases have been implicated as important contributors to this process. They are known to release NO₂⁻ from nitroglycerin, but have not been shown to release NO[•]. The present studies were designed to examine the role of endogenous glutathione S-transferases in this metabolic process. Homogenates of dog carotid artery were incubated anaerobically with nitroglycerin, and NO[•] and NO₂⁻ production was determined by chemiluminescence. The role of glutathione S-transferases was studied by incubating homogenates with nitroglycerin in the presence of 1 mM GSH or 1 mM S-hexylglutathione, a potent inhibitor of glutathione S-transferases. Homogenates released 163 pmol of NO[•]/h per mg of protein from nitroglycerin, and 2370 pmol of NO₂⁻/h per mg. Adding

GSH decreased NO[•] production by 82% and increased NO₂⁻ production by 98%. S-Hexylglutathione inhibited glutathione S-transferase activity by 96% and decreased NO₂⁻ production by 78%, but had no effect on NO[•] release. A linear relationship between glutathione S-transferase activity and NO₂⁻ production was observed, whereas glutathione S-transferase activity and NO[•] release were unrelated. Western-blot analysis demonstrated that dog carotid vascular smooth muscle contained Pi and Mu forms of glutathione S-transferases, with a predominance of the former. Purified preparations of human Pi and rat Mu isoforms metabolized nitroglycerin only to NO₂⁻ and not to NO[•]. On the basis of these findings, we conclude that (1) glutathione S-transferases do not contribute to the bioconversion of nitroglycerin to NO[•], but instead act as a degradative pathway for nitroglycerin, and (2) the release of NO[•] from nitroglycerin is not dependent on the formation of NO₂⁻.

INTRODUCTION

Despite extensive clinical use, the precise mechanism by which nitroglycerin causes vasodilatation remains unclear. It has no direct vasodilator effect of its own, but must be converted by vascular smooth muscle into an active vasodilator species, probably NO[•], or a related compound [1–6]. The mechanisms responsible for this bioactivation are not well understood. Both enzymic and non-enzymic processes have been implicated.

Recent evidence suggests that the conversion of nitroglycerin into its vasodilator metabolite is an intracellular enzymic process [7–11]. One enzyme system implicated in this process is the glutathione S-transferases [10,11]. The glutathione S-transferases (EC 2.5.1.18) are a family of enzymes present in the cytosol of most cells [12,13]. The mammalian enzymes can be grouped into four classes termed Alpha, Mu, Pi and Theta based on their functional and structural properties [14,15]. Several isoenzymes of hepatic glutathione S-transferases have been found to convert nitroglycerin into glyceryl dinitrate and NO₂⁻ [10,11,16,17]. Recently, glutathione S-transferase isoenzymes similar to those of the Alpha and Mu classes isolated from vascular tissues were shown to metabolize nitroglycerin to dinitrate and NO₂⁻ [18]. It has been suggested that this release of NO₂⁻ by glutathione S-transferase may be an important initial step in the production of NO[•] from nitroglycerin [19]. The ability of NO₂⁻ to serve as an intermediate in the formation of NO[•] from nitroglycerin, however, has been questioned because NO₂⁻ produces vasodilatation only at very high concentrations, and formation of NO[•] from NO₂⁻ is not favoured at neutral pH. Another possibility is that glutathione S-transferases either directly or via an intermediate yield NO[•] from nitroglycerin. In support of this hypothesis,

Yeates et al. [20] have reported that sulphobromophthalein, an inhibitor of glutathione S-transferases, diminishes the vasodilator effect of nitroglycerin in rabbit aorta.

In the present study, we examined the conversion of nitroglycerin into NO[•] and NO₂⁻ in vascular tissues. We sought to determine the role of the intrinsic vascular smooth-muscle glutathione S-transferases in the metabolism of nitroglycerin to NO[•]. Studies were performed in homogenates of vascular tissue so that the reaction environment could be readily manipulated. Specific interventions were employed to augment and inhibit the activity of the intrinsic glutathione S-transferases. Finally, studies were performed to determine relative amounts of NO[•] and NO₂⁻ produced on the metabolism of nitroglycerin by purified glutathione S-transferases.

MATERIALS AND METHODS

Preparation of vascular homogenate

Dogs were anaesthetized with pentobarbital sodium (75 mg/kg), heparinized (1000 units/kg) and killed with KCl. The carotid arteries were removed within 5 min of death and placed in ice-cold Krebs'/bicarbonate buffer for transport to the laboratory. All further preparation was carried out on ice. Vessels were placed in PBS (pH 7.3) containing (mM) NaCl (140), KCl (3), K₂HPO₄ (6.6) and KH₂PO₄ (5), and thoroughly cleaned of adventitia. The vessels were then opened longitudinally and the luminal surface rubbed to remove the endothelium. The remaining tissue, containing primarily vascular smooth muscle, was finely minced with scissors and homogenized in a glass tissue homogenizer. The resulting suspension was further subjected to three 20 s bursts with an ultrasonic cell disrupter. This

Abbreviations used: IEF, isoelectric focusing; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

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homogenate was then centrifuged at low speed (680 *g*) to remove any remaining whole cells. The pellet was discarded, and the supernatant diluted with PBS to a total volume of about 10 ml. This produced a vascular tissue homogenate with an average final protein concentration of 2.0 ± 0.2 mg/ml. The homogenate was deoxygenated in a sealed 125 mm $\times$ 16 mm vacutainer-type tube by purging with argon for a minimum of 45 min. In separate experiments, a portion of the homogenate was heated to 100 °C for 15 min before purging with argon.

Western-blot analysis of glutathione S-transferases and isoelectric focusing

Western blots were performed using standard techniques. Glutathione S-transferase antibodies were purchased from Bioprep (Dublin, Ireland). Anti-(rabbit IgG) antibody labelled with alkaline phosphatase was purchased from Bio-Rad. Isoelectric focusing (IEF) was performed using the Bio-Rad mini-IEF cell according to the manufacturer's instructions.

Reagent preparation

GSH was prepared immediately before use by dissolving it in 0.5 M phosphate buffer (pH 7.2) to produce a 100 mM stock solution. *S*-Hexylglutathione was dissolved in deionized water at pH 10.0. Phosphate buffer was then added to produce a 20 mM stock solution at pH 7.2. This stock solution was then stored in portions at -70 °C. Nitroglycerin was prepared daily by diluting the stock solution with phosphate buffer to a final concentration of 10 mM. This solution was placed in a sealed 1.5 ml amber vial and purged with argon for 3 min to remove oxygen. All reagents were purchased from Sigma except nitroglycerin which was obtained from Dupont.

Preparation of purified glutathione S-transferase

Glutathione S-transferase (Yb₂Yb₂) was purified from rat liver using a combination of affinity chromatography and chromatofocusing [21,22]. The purified isoform yielded a single band on SDS/PAGE, cross-reacted with specific anti-(glutathione S-transferase) sera, and had a pI of 7.2 on IEF. Human glutathione S-transferase Pi was produced in and purified from bacteria transfected with a plasmid containing the complete human glutathione S-transferase-coding sequence [23].

Experimental protocols

To assess the metabolism of nitroglycerin by vascular tissue, homogenates of carotid artery were incubated with nitroglycerin for 1 h in 1.5 ml amber glass microreaction vials. All components except homogenate and nitroglycerin were added to the vials. The vials were then sealed with aluminium caps with Teflon septa and purged with argon for 3 min. Deoxygenated homogenates were then added to the appropriate vials, and finally, nitroglycerin was added. Each vial thus contained 400 μ l of carotid homogenate and 0.1 μ M nitroglycerin. In some experiments, either 1 mM of *S*-hexylglutathione or 1 mM GSH was added. Sufficient PBS was added to bring the final reaction volume to 500 μ l. The vials containing reaction mixtures were then placed in an incubator (37 °C) equipped with a shaker for 1 h.

Studies of purified glutathione S-transferases

Separate experiments were performed in which purified preparations of either the Mu or Pi form of glutathione S-transferase were incubated with nitroglycerin (100 μ M) and GSH (1 mM) to determine if these could directly release NO[•] from nitroglycerin.

Figure 1 Schematic of system for measuring NO[•] and NO₂⁻

Nitrogen is continuously drawn by vacuum through the chemiluminescence NO[•] detector. A valve allows by-passing or directing of nitrogen into the microreaction vial and through the reaction mixture (inset). Any NO[•] present is drawn with nitrogen into the detector. After all NO[•] is purged from the vial, a trichloroacetic acid/NaI is added to reduce NO₂⁻ to NO[•]. The vial is then purged a second time for determination of NO₂⁻ production.

Measurement of nitrogen oxides

NO[•] and NO₂⁻ released during the metabolism of nitroglycerin by vascular homogenates were measured using a chemiluminescence NO[•] analyser (Dasibi, Glendale, CA, U.S.A.). NO[•] gas in the headspace and in solution was measured by flushing individual vials with N₂ using a preparation schematically illustrated in Figure 1. N₂ was drawn via a vacuum through one needle into the reaction mixture, and the purged gasses were then directed through a second needle directly into the NO[•] analyser. After NO[•] gas production had been determined for all samples, 200 μ l of a solution containing 10% trichloroacetic acid and 10% NaI was added to each vial. This acidification/reduction solution causes a rapid one-electron reduction of NO₂⁻ to NO[•] gas, which could be measured by the chemiluminescence detection device. Each vial was then purged with N₂ for a second occasion as described above. NaI was added to the acidification solution to increase the rate of conversion of NO₂⁻ into NO[•]. Since this reducing agent also reduces nitrosothiols to NO[•], it is conceivable that if these compounds were present in the vascular homogenates, they could have been falsely identified as NO₂⁻. Nitrosothiols are stable at low pH, whereas NO₂⁻ is converted into NO[•]. Thus separate experiments were performed in which trichloroacetic acid was added to the reaction mixtures and NO[•] was sampled before the addition of NaI. In these studies, no further NO[•] was released by adding NaI to acidified homogenate solutions. This finding indicates that either nitrosothiols are not formed during bioconversion of nitroglycerin or were reduced to free NO[•] before measurement. Thus the use of NaI to speed the release of NO[•] from NO₂⁻ did not affect the interpretation of the present findings.

The NO[•] analyser was calibrated with known concentrations of NO₂⁻ in microreaction vials treated with trichloroacetic acid/NaI. A standard curve was generated each day which encompassed the range of nitrogen oxides produced by the experimental samples. The standard curve was linear over this entire range (25–500 pmol, $r^2 = 0.97$). The lower limit of the detection of this assay was 10 pmol of NO[•]. An example of an experimental record is shown in Figure 2.

Determination of glutathione S-transferase activity and GSH levels

Glutathione S-transferase activity was determined by spectrophotometric assay with 1-chloro-2,4-dinitrobenzene (CDNB),

Figure 2 Example of an experimental record

The first three peaks were produced by acidified NO₂⁻ solutions (standards) of known concentrations. Following are peaks produced by dog carotid artery homogenates alone (CAH), in the presence of nitroglycerin (CAH + NTG), nitroglycerin and GSH (CAH + NTG + GSH) and nitroglycerin, GSH and S-hexylglutathione (CAH + NTG + S-HxGSH). The final peak is NO[•] produced by incubation of nitroglycerin with GSH in PBS. This peak is not significantly different from that produced by phosphate buffer alone (injection artifact).

ethacrynic acid, cumene hydroperoxide or 1,2-dichloro-4-nitrobenzene (DCNB) using a recording spectrophotometer as described previously [21,24]. Homogenate (500 µg approximate protein content) was added to the cuvette at $t = 0$. The maximal rate (typically obtained within 30 s of homogenate introduction) of formation was used as the index of glutathione S-transferase activity. GSH was measured in vascular homogenates as described previously [25,26].

Data analysis

Vials were prepared in triplicate, and the average nitrogen oxide production for the three vials was taken as the response for that experiment. For all experiments, negative controls (homogenate without nitroglycerin) were performed, and any nitrogen oxides detected in these samples were subtracted from the samples incubated with nitroglycerin. In experiments that included the addition of GSH to homogenates (or to purified glutathione S-transferase), an additional negative control (GSH + nitroglycerin in PBS) was also performed to determine what portion of the release of nitrogen oxides from nitroglycerin could be attributed to non-enzymic mechanisms. NO[•] was not produced in measurable quantity by the non-enzymic interaction of nitroglycerin and GSH in PBS, but NO₂⁻ was consistently detected in these samples. The amount of NO₂⁻ formed by the non-enzymic reaction of GSH and nitroglycerin averaged 89 ± 83 pmol/reaction vial. In each experiment, this non-enzymic value was subtracted from that produced by homogenates incubated with nitroglycerin and GSH. Thus only nitrogen oxides that could be directly attributed to the metabolism of nitroglycerin by vascular tissue were studied.

Nitrogen oxide production (both NO[•] and NO₂⁻) by samples treated with GSH or S-hexylglutathione was compared with that

in paired controls using Student's paired t test. Differences were considered statistically significant at $P \leq 0.05$. In most cases, results are means $\pm$ S.E.M.

RESULTS

Glutathione S-transferase and GSH in vascular homogenates

The predominant form of glutathione S-transferase in dog carotid artery was the Pi form. Mu form(s) was also present, but no Alpha-class glutathione S-transferases were identified (Figures 3b and 3c). Positive reactions with all three antibodies were obtained when the 100 000 g supernatant of dog liver was examined, demonstrating that the rat antibodies could detect the dog isoforms adequately. Enzymic activity assessed by the various assays was: with CDNB, 0.16 µmol/min per mg; DCNB, none; ethacrynic acid, 0.01 µmol/min per mg; cumene hydroperoxide, 0.044 µmol/min per mg. The vascular homogenates contained

Figure 3 Western-blot analysis of cytosol from dog vascular smooth muscle (M) and liver (L)

Cytosolic protein (15 µg) was added to lanes M and L. Human glutathione S-transferase Pi (2 µg) was added to lanes Pi. Anti-Pi (a), anti-Mu (b) and anti-Alpha (c) sera were used as primary antibodies. M_r standards (St) were 30 000 (closed arrow) and 22 000 (open arrow). Anti-Alpha sera cross-react weakly with glutathione S-transferase Pi.

Figure 4 $\text{NO}^\bullet$ production (a) and NO_2^- production (b) from metabolism of nitroglycerin by vascular homogenates under control conditions, in the presence of 1 mM GSH, 1 mM *S*-hexylglutathione (*S*-HxGSH) and heat-denatured homogenates (HDH)

*Significantly different from control, $P < 0.05$.

Figure 5 Relationship between glutathione *S*-transferase activity and $\text{NO}^\bullet$ and NO_2^- production from nitroglycerin by vascular homogenates

A highly significant correlation existed between glutathione *S*-transferase activity and NO_2^- production (●, $r^2 = 0.88$). $\text{NO}^\bullet$ production was unrelated to glutathione *S*-transferase activity (△, $r^2 = 0.12$).

2.2 ± 0.3 nmol of GSH of protein. Therefore in the absence of any added GSH, the approximate concentration of GSH in the $\text{NO}^\bullet/\text{NO}_2^-$ assay was $3.9 \mu\text{M}$.

Production of $\text{NO}^\bullet/\text{NO}_2^-$ by vascular homogenates under control conditions

Homogenates of canine carotid artery released $\text{NO}^\bullet$ gas from nitroglycerin at a rate of 163 ± 25 pmol/h per mg under control conditions. NO_2^- was also a product of nitroglycerin metabolism, appearing at a rate of 2370 ± 682 pmol/h per mg. Heating to 100°C for 15 min decreased the release of $\text{NO}^\bullet$ and NO_2^- from nitroglycerin by 96% and 85% respectively (Figure 4).

Treatment of homogenates with *S*-hexylglutathione decreased glutathione *S*-transferase activity assayed with either ethacrynic acid or CDNB by more than 95%, but had no effect on the bioconversion of nitroglycerin to free $\text{NO}^\bullet$ (Figure 4). Conversely, addition of the *S*-hexylglutathione to the vascular homogenates decreased the release of NO_2^- from nitroglycerin by $78 \pm 7\%$ (Figure 4).

Addition of 1 mM GSH to the reaction mixture significantly decreased the release of $\text{NO}^\bullet$ gas from nitroglycerin ($-82 \pm 9\%$, $P < 0.05$, Figure 4) but greatly increased the rate of NO_2^- formation ($+98 \pm 19\%$, $P < 0.05$, Figure 4).

Relationship between glutathione *S*-transferase activity, $\text{NO}^\bullet$ and NO_2^- production

Glutathione *S*-transferase activity (assayed with CDNB) in control homogenate preparations varied from 30 to 204 nmol/min per mg (mean $\pm$ S.E.M. = 107 ± 25). Simple regression analysis revealed a highly significant correlation between glutathione *S*-transferase activity and NO_2^- release from nitroglycerin ($r^2 = 0.88$, Figure 5). No such correlation existed between glutathione *S*-transferase activity and $\text{NO}^\bullet$ release ($r^2 = 0.12$, Figure 5).

Effect of purified glutathione *S*-transferase on nitroglycerin

The activities of the preparations of the Mu and Pi forms of glutathione *S*-transferases within the reaction vials, as assessed by the CDNB assay, were 5.8 nmol/min per mg and 440 nmol/min per mg respectively. In the presence of 1 mM GSH, both the rat Mu and the human Pi forms of glutathione *S*-transferase released NO_2^- . Interestingly, the specific activity of the Mu form toward nitroglycerin was approx. 10-fold that of the Pi form (Mu = 0.2 versus Pi = 0.02 $\mu\text{mol}/\text{min}$ per mg of protein), a finding in direct contrast with the specific activities observed when CDNB was employed as a substrate. Neither isoform released $\text{NO}^\bullet$ in quantities above the limit of detection.

DISCUSSION

$\text{NO}^\bullet$ or an $\text{NO}^\bullet$ -containing compound is generally accepted to be the active vasodilator metabolite of nitroglycerin. Several previous studies have shown that glutathione *S*-transferases metabolize nitroglycerin to glyceryl dinitrate and NO_2^- [10,11,16,17]. These findings suggest that glutathione *S*-transferases are important mediators of the conversion of nitroglycerin into $\text{NO}^\bullet$ (via the release of NO_2^- as an intermediate) [10,11,16–19]. In none of these studies, however, was $\text{NO}^\bullet$ measured directly. The present findings demonstrate that both $\text{NO}^\bullet$ and NO_2^- are released during the metabolism of nitroglycerin by vascular tissue. Metabolism is probably dependent on one or more enzyme systems, since the release of both $\text{NO}^\bullet$ and NO_2^- was virtually eliminated by heating vascular homogenates to 100°C .

We believe that the glutathione *S*-transferases are not involved in the bioactivation of nitroglycerin because of the following observations. (1) Purified preparations of the Mu and Pi forms of glutathione *S*-transferase produced large amounts of NO_2^- from nitroglycerin, but little $\text{NO}^\bullet$. In fact, all of the $\text{NO}^\bullet$ produced could be accounted for by the equilibrium that forms between NO_2^- and $\text{NO}^\bullet$ when the former is in solution ($\text{pK}_a = 4 \times 10^{-3}$). (2) Although a linear relationship between glutathione *S*-transferase activity and NO_2^- production was observed, no such relationship existed for the production of $\text{NO}^\bullet$. (3) Addition of *S*-hexylglutathione, a potent inhibitor of glutathione *S*-transferases, had no effect on $\text{NO}^\bullet$ release from nitroglycerin, whereas addition of GSH to the vascular homogenates, which would be expected to increase glutathione *S*-transferase activity, actually decreased $\text{NO}^\bullet$ release. (4) Although several studies have suggested that the first intermediate product in the bioactivation of nitroglycerin is NO_2^- [1,2,19,27], this seems to be unlikely given the relatively weak vasodilator properties of NO_2^- and the limited conversion

of NO_2^- into NO^* at neutral pH. Further, if NO_2^- were a required intermediate in the formation of NO^* , increases in NO_2^- production from nitroglycerin would be expected to increase NO^* release. In contrast, the present findings demonstrate that manipulations that increased NO_2^- production were associated with decreases in NO^* production.

An important conclusion from the present studies is that the glutathione S-transferases, rather than playing a role in the bioactivation of nitroglycerin, instead appear to play a degradative role. For example, increasing glutathione S-transferase activity by adding GSH increased production of NO_2^- but decreased production of NO^* , indicating that glutathione S-transferases only metabolize nitroglycerin to NO_2^- . A second as yet unidentified system appears to metabolize nitroglycerin to NO^* . This alternative pathway does not appear to be the microsomal form of glutathione S-transferase [28]. The finding that inhibition of glutathione S-transferases did not increase NO^* production from nitroglycerin is at odds with a competitive interaction between the glutathione S-transferases and putative enzyme systems responsible for NO^* production from nitroglycerin. This apparent paradox could be explained if the enzyme system responsible for the conversion of nitroglycerin into NO^* were operating near V_{max} . Under these conditions, removal of a competitive pathway would have minimal effect.

By Western-blot analysis, dog aorta smooth muscle contains Mu and Pi but not Alpha forms of glutathione S-transferase. Although we did not purify the glutathione S-transferases from dog aorta, we examined metabolism of nitroglycerin by Mu and Pi forms isolated from rat and man respectively. Given the similar substrate specificities of the various isoforms irrespective of species [12], this approach appears justified. CDNB and nitroglycerin, as shown in the current study, are both good substrates for Mu- and Pi-class enzymes. Ethacrynic acid is an excellent substrate for Pi-class enzymes [12]. Thus the glutathione S-transferase activities present in dog vascular smooth muscle cell cytosol are consistent with the findings obtained by Western-blot analysis. Theta-class enzymes have little activity with CDNB, do not bind to GSH or *S*-hexylglutathione affinity columns and may not be inhibited by *S*-hexylglutathione [15]. Therefore, Theta-class enzymes could have been present in the vascular cytosol and not recognized by the current analysis. However, there is an excellent correlation between glutathione S-transferase activity, when assayed with CDNB, and NO_2^- release, suggesting that the potential contribution of any Theta-class enzymes to the metabolism of nitroglycerin in vascular smooth muscle is small.

The present studies were performed under anaerobic conditions. This was essential to minimize the oxidation of any NO^* formed from nitroglycerin. It is conceivable that the capacity of the vascular wall to form NO^* from nitroglycerin may be substantially different in an aerobic environment. The glutathione S-transferases, however, are not dependent on molecular oxygen. It would seem that the conditions of our assays optimized the likelihood that NO^* be formed by the interactions of the glutathione S-transferases and nitroglycerin.

The decrease in NO^* release from nitroglycerin in vascular smooth muscle when exogenous GSH was added might be attributed to generation of superoxide anion by GSH within the reaction vial [29]. The reaction of superoxide anion and NO^* could contribute to the increase in NO_2^- observed via a peroxynitrite intermediate. This explanation for our findings seems very unlikely for several reasons. First, the reaction occurred in anoxic conditions, such that the amount of molecular oxygen available for univalent reduction would be minimal. Secondly, the reaction mixture contained a relatively high concentration of superoxide dismutase, which would minimize the presence of

superoxide anion. Finally, the increase in NO_2^- in the presence of GSH greatly exceeded the amount of NO^* produced under control conditions, and it therefore is impossible to account for the increased NO_2^- production in the presence of GSH by conversion of the relatively small amounts of NO^* into NO_2^- .

The cytochrome *P*-450s or related reductases have been suggested to play an important role in the bioactivation of nitroglycerin. This concept is supported by several lines of evidence, including the finding that nitroglycerin is metabolized to free NO^* by the microsomal fraction of vascular tissue homogenates [30]. It has also been clearly demonstrated that cytochrome *P*-450s can metabolize nitroglycerin to dinitrate and NO_2^- metabolites [31]. The interaction between cytochrome *P*-450 and nitroglycerin is strongly inhibited by oxygen, however, and so the importance of this enzyme system in nitroglycerin bioactivation remains controversial.

In conclusion, the present studies demonstrate that glutathione S-transferases present in vascular tissue do not contribute significantly to the bioconversion of nitroglycerin to NO^* . The data suggest that the glutathione S-transferases may in fact represent a competitive pathway for the metabolism of nitroglycerin by converting nitroglycerin into non-vasoactive NO_2^- and glyceryl dinitrate. Finally, the present findings directly demonstrate that the formation of NO^* from nitroglycerin is not related to the formation of NO_2^- . The enzyme(s) responsible for the bioactivation of nitroglycerin has (have) yet to be identified.

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