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(54) Title: A METHOD OF REDUCING A VASODILATORY RESPONSE USING A GUANYLATE CYCLASE PATHWAY INHIBITOR OR AN H₂O₂ REDUCING AGENT

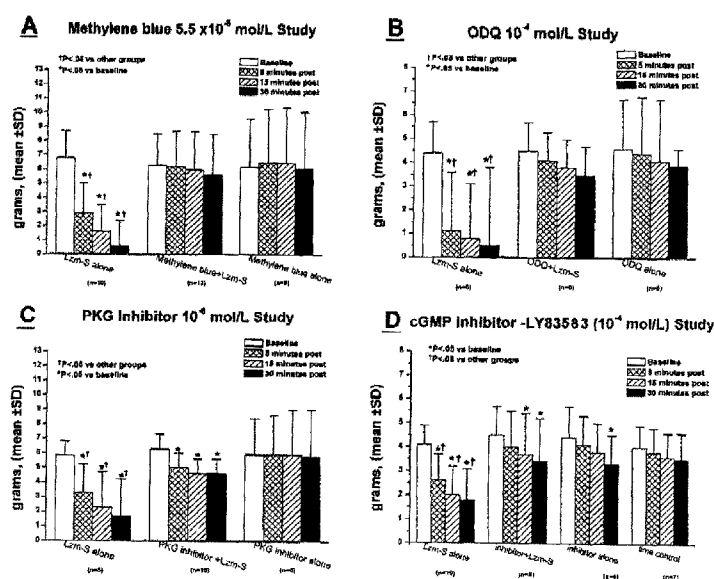


Figure 5

(57) Abstract: A method of reducing a vasodilatory response comprising administering to an individual in need of such treatment an effective amount of a guanylate cyclase pathway inhibitor selected from the group consisting of methylene blue, ODQ, LY83583 and a protein kinase G inhibitor or an H₂O₂ reducing agent selected from the group consisting of Aspergillus niger catalase and hydroxyl scavengers, hydroquinone and mannitol.

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A method of reducing a vasodilatory response using a guanylate cyclase pathway inhibitor or an H₂O₂ reducing agent.

PRIOR APPLICATION INFORMATION

The instant application claims the benefit of US Provisional Patent Application
5 60/968,391, filed August 28, 2007.

BACKGROUND OF THE INVENTION

Septic shock is a clinical syndrome that results from an activated systemic host inflammatory response to infection leading to cardiovascular collapse. (1) Widespread
10 systemic vasodilation and a decrease in myocardial contractility contribute to the hypotension observed in this condition (1,25,26,28). Both components reflect the effects of mediators that are released as part of the inflammatory response. The recruitment of leukocytes into sites of inflammation leads to interaction with the vascular endothelium and promotes the release of a wide range of proinflammatory
15 cytokines, such as interleukin-6 and tumor necrosis factor- α among other classes of mediators (12,28). Many of these mediators are associated with the release of nitric oxide (NO) by activation of endothelial and inducible nitric oxide synthases. NO is thought to play a prominent role in the vasodilation that accompanies septic shock (10).

20 We previously found that lysozyme (Lzm-S), released from leukocytes contributed to the myocardial depression that develops in septic shock (13,19-21). We showed that the mechanism by which Lzm-S caused myocardial depression was related to the binding of its catalytic site to the endocardial endothelium (EE) (19). This interaction resulted in the release of NO, the effect of which could be inhibited by
25 the non-specific nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA). After release of NO from the EE, NO diffuses to adjacent myocytes to activate the guanosine 3', 5' monophosphate (cGMP) pathway. cGMP may then depress contraction by multiple mechanisms that include among others a reduction in myofilament Ca²⁺ responsiveness via activation of cGMP-dependent protein kinase
30 (PKG), and an inhibition of myocardial calcium channel activity (17).

Since Lzm-S causes NO release by interaction with the EE, we hypothesized that Lzm-S may also contribute to the systemic vasodilation observed in septic shock by binding to the endothelium of the systemic vasculature. In the present study, we used a phenylephrine contracted carotid artery ring preparation to examine the extent to which Lzm-S may contribute to a reduction in vasculature tone in septic shock. We examined whether release of NO by the endothelium or whether other mechanisms contributed to Lzm-S's vasodilatory response.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method of reducing a vasodilatory response comprising administering to an individual in need of such treatment an effective amount of a guanylate cyclase pathway inhibitor or an H_2O_2 reducing agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. In the examples shown, canine lysozyme was placed into the phenylephrine constricted carotid ring preparation in Panel A, while human lysozyme was placed into the preparation in Panel B. There was a decrease in constriction to about 40% of the phenylephrine value in both conditions.

Figure 2. In the graphs shown, the mean values are shown for the canine lysozyme experiment in Panel A and for the human lysozyme experiment in Panel B. Both lysozyme preparations caused significant vasorelaxation in the carotid artery preparation.

Figure 3. Examples in which treatment with the non-specific nitric oxide inhibitor L-NMMA (N^G -monomethyl-L-arginine) (Panel A) and removal of the endothelium by mechanical denudation (Panel B), respectively, did not inhibit lysozyme induced vasodilation.

Figure 4. In the example shown, incubation with methylene blue inhibited the effect of lysozyme.

Figure 5. Inhibitors of the guanosine 3', 5' monophosphate (cGMP) pathway prevented lysozyme induced vasodilation. These inhibitors included methylene blue (Panel A), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) (Panel B), the protein kinase G inhibitor guanosine 3',5' -cyclic monophosphorothioate, β -phenyl-1,N²-etheno-8-bromo-Rp-isomer, sodium salt (Panel C), and LY 83583 (6-anilino-5,8-quinolinequinone) (Panel D).

Figure 6. In the example shown in Panel A, the peroxidase metabolizing enzyme *Aspergillus niger* catalase inhibited the effect of lysozyme. The mean results are shown in Panel B.

Figure 7. In the example shown in Panel A, ethanol inhibited the effect of lysozyme. The mean results found at various ethanol concentration are shown in Panel B.

Figure 8. In Panel A, the effect of H₂O₂ on force in the carotid preparation is shown. For the higher concentrations of H₂O₂, there was an initial increase in force that was followed by vasorelaxation. For the lower concentrations, vasorelaxation was produced. The mean results are shown in Panel B.

Figure 9. Both the hydroxyl radical scavengers (mannitol) (Panel A) and hydroquinone (hyd) (Panel B) inhibited the vasodilatory effect of lysozyme in the carotid artery preparation.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

In septic shock, systemic vasodilation and myocardial depression contribute to the systemic hypotension observed. Both components reflect the effects of mediators that are released as part of the inflammatory response. We previously found that lysozyme (Lzm-S), released from leukocytes, contributed to the myocardial

depression that develops in a canine model of septic shock. Lzm-S binds to the endocardial endothelium releasing nitric oxide (NO) that in turn activates the myocardial guanylate cyclase (sGC) pathway. In the present study, we determined whether Lzm-S may also contribute to the systemic vasodilation that is found in septic shock. In a phenylephrine contracted canine carotid artery ring preparation, we found that both canine and human Lzm-S, at concentrations similar to those found in sepsis, produced vasorelaxation. This relaxation could not be prevented by inhibitors of nitric oxide synthase, prostaglandin synthesis, or potassium channel inhibitors and was not dependent on the presence of the vascular endothelium. However, inhibitors of guanylate cyclase (sGC) pathway that included methylene blue, ODQ, LY83583, and a protein kinase G inhibitor all prevented Lzm-S's vasodilatory effect. In addition, the peroxide metabolizing enzyme, *Aspergillus niger* catalase, that would breakdown H_2O_2 as well as the hydroxyl scavengers that included hydroquinone and mannitol prevented Lzm-S's effect. These results are consistent with the notion that when Lzm-S interacts with the smooth muscle membrane, this interaction results in the formation of H_2O_2 that in turn activates the sGC pathway to cause vasorelaxation. This pathway may contribute to the vasodilation that occurs in septic shock.

Lzm-S is a newly discovered inflammatory mediator that has been shown to cause myocardial depression in an *E coli* experimental model of sepsis (20). In the present study, we examined the extent to which Lzm-S may contribute to the systemic vasodilation that is also observed in this model. The concentrations of Lzm-S used in this study were comparable to those previously found to occur after 4 to 6 hours of *E coli* bacteremia in dogs (20). In the present study, the results showed that both canine and human Lzm-S produced significant vasorelaxation in a phenylephrine contracted carotid artery ring preparation.

Based on our previous cardiac experiments (13, 19-21), we initially considered that the release of mediators from the endothelium, particularly NO, was the mechanism by which Lzm-S caused this vasodilatory effect. Nevertheless, when we tested for the role of NO by pre-treatment of the carotid ring with L-NMMA, we did not find that Lzm-S-induced vasorelaxation was inhibited. We further tested for

commonly known pathways of vasodilation that included blockade of the prostaglandin pathway with indomethacin, blockade of the bradykinin β_1 and β_2 receptors by B9340, inhibition of adenylyl cyclase production by SQ 22, 536, inhibition of potassium channels, as well inhibition by the β -adrenergic receptor antagonist propranolol and the muscarinic receptor antagonist atropine (6, 11, 17, 30). In addition, when we removed the carotid artery endothelium, we found that the vasodilatory effect of Lzm-S was still present. Thus, the release of mediators from the endothelium or the aforementioned pathways did not contribute to the vasorelaxation caused by Lzm-S.

We also determined whether inhibitors of Lzm-S's catalytic site, namely chitobiose and chitotriose, would prevent Lzm-S-induced vasorelaxation (21, 27). In the heart, the binding of Lzm-S to a N-glycoprotein on the endocardial endothelium mediates the release of NO (19). Lzm-S specifically binds to the Man $\beta(1-4)$ GlcNAc $\beta(1-4)$ GlcNAc moiety in the tri-mannosyl core structure of high mannose/hybrid and tri-antennary carbohydrate classes where GlcNAc is N-acetylglucosamine and Man is mannose (13). The Man $\beta(1-4)$ GlcNAc $\beta(1-4)$ GlcNAc moiety is structurally similar to chitobiose and chitotriose compounds, and these two agents have been shown to competitively inhibit Lzm-S's myocardial depressant effect in in-vivo and in-vitro preparations (13, 19-21), although the receptor to which Lzm-S binds is not clear. Thus, the signaling pathway and the critical binding site of Lzm-S with respect to vasorelaxation are distinctly different from the mechanisms that contribute to myocardial depression caused by this mediator.

Since removal of the endothelium did not inhibit Lzm-S's vasodilatory effect, the binding of Lzm-S to the smooth muscle cell rather than the endothelium resulted in the vasorelaxation observed. Moreover, this binding of Lzm-S to the smooth muscle membrane resulted in activation of the cGMP pathway, as evidenced by the fact that inhibitors of this pathway prevented Lzm-S's effect. Although activation of the cGMP pathway represented the apparent 2nd messenger system for vasorelaxation, the mechanism by which this pathway was activated was not initially clear, since none of the commonly noted pathways, usually associated with cGMP

activation, were able to block Lzm-S's effect. We therefore looked for other possibilities.

After many screening experiments were performed, we found that ethanol was able to inhibit Lzm-S's vasodilatory effect. We therefore focused on the experiments by the laboratory of Wolin and colleagues (2,3). In a precontracted bovine pulmonary artery preparation, these investigators showed that H_2O_2 mediates vasorelaxation and that both methylene blue and ethanol inhibited this effect. Ethanol is an agent that is known to modulate peroxide metabolism through endogenous catalase. Ethanol is associated with a decrease in the Compound I species that would in turn cause a reduction in cGMP thereby inhibiting vasodilation. In the present study, we hypothesized that the binding of Lzm-S to the smooth muscle cell might activate H_2O_2 and furthermore that this effect may be inhibited by both a peroxidase and ethanol. Since the results showed that both these agents blocked Lzm-S's vasodilatory effect, these findings would support the hypotheses of this study.

We also showed that exogenous H_2O_2 produced vasodilation in the carotid artery preparation. At the higher H_2O_2 concentrations that ranged between 10^{-3} to 10^{-1} mol/L, there was a biphasic time response to H_2O_2 in which the initial response was a contraction at 5 minutes-post instillation that was followed by a vasodilator response at 30 minutes-post instillation. As concentrations of H_2O_2 were increased, we found that the relative magnitudes of both vasodilation and vasoconstriction increased, while at a concentration of 10^{-4} mol/L, the response was one of vasodilation alone. Burke and Wolin (2) also found a biphasic dose- response effect of exogenous H_2O_2 administration in a precontracted bovine pulmonary artery preparation, in which relative vasodilation increased as concentrations were raised from 10^{-6} to 10^{-4} mol/L after which vasorelaxation started to decrease at 10^{-3} mol/L. We think that both studies support the notion that exogenous H_2O_2 may activate multiple compartmental cellular pathways, since there were biphasic responses in both studies. Although the net effect on vasculature tone would depend on the extent to which each of these

pathways was activated, the present study shows that Lzm-S produced a vasodilatory response in this carotid preparation.

We conclude that the present results are consistent with the notion that the interaction of Lzm-S with the vascular smooth muscle membrane results in the generation of H_2O_2 that in turn activates the cGMP pathway to cause vasorelaxation. Although generated extracellularly, H_2O_2 may initiate intracellular signaling pathways resulting in the activation of cGMP (8, 22). cGMP would then cause vasodilation by altering myofilament responsiveness to calcium or by inhibiting calcium entry into the cell (17).

The pathways by which H_2O_2 may be generated under different conditions are complex and not completely understood (5), and it is not yet clear the mechanism by which Lzm-S would generate H_2O_2 . The potential enzymatic sources of reactive oxygen species, such as H_2O_2 include mitochondrial electron transport chain, the arachidonic acid metabolizing enzymes lipoxygenase and cyclooxygenase, NAD(P)H oxidases, and other hemoproteins, among others. A membrane location for Lzm-S's site of H_2O_2 generation appears likely, since its 17,000 molecular weight would make transport to an intracellularly location less feasible. The majority of the bioactive H_2O_2 is derived from spontaneous or SOD catalyzed dismutation of $O_2^{\cdot -}$ (5). One electron reduction of molecular oxygen forms superoxide anion ($O_2^{\cdot -}$). Among the possible mechanisms to consider, we initially thought that H_2O_2 would be derived from superoxide with SOD, since this mechanism has been shown to activate guanylate cyclase in other experiments (7). However, we could not prevent Lzm-S's vasodilator effect with the SOD inhibitor DECTA, so that we do not think that this enzyme system was involved in the vasodilation observed.

Another mechanism to consider is that some proteins and antibodies may be capable of producing H_2O_2 under certain conditions. DeYulia et al (8) found that H_2O_2 could be generated by specific receptor-ligand interaction in cells and in cell-free systems. They showed that the extracellular domain of hematopoietin receptors interacting with cognate ligand is sufficient to generate H_2O_2 , although the mechanism of this generation was not well defined. In other studies, Wentworth and colleagues

(32-34) reported that proteins, such as antibodies may generate H_2O_2 from singlet molecular oxygen ($^1\text{O}_2^*$) and water; moreover, that this generation may occur regardless of the antigen specificity of the antibody. They suggest that some proteins, particularly antibodies have the intrinsic ability to intercept $^1\text{O}_2^*$ and efficiently reduce it to $\text{O}_2^{\cdot-}$ which then dismutates spontaneously into H_2O_2 . Although the pathways have not been completely elucidated, we propose that a protein such as Lzm-S also processes the property to generate H_2O_2 in a manner described by Wentworth and colleagues (32-34). We further suggest that this is the initiating pathway by which vasodilation occurred in our preparation.

In the present study, we additionally showed that the hydroxyl radical scavengers mannitol and hydroquinone prevented Lzm-S's vasodilatory effect. Thus, in addition in H_2O_2 , the formation of the hydroxyl radical appears to be an important component of the pathway that contributes to Lzm-S's effect. The blockade of Lzm-S-induced vasodilation by mannitol and hydroquinone was less effective (60% vs 90%) as compared to what was observed with the peroxidase from *Aspergillus niger*, so that the generation of the hydroxyl radical may play a lesser role than that of the H_2O_2 molecule in producing the vasodilation caused by Lzm-S. We also administered the iron chelator deferoxamine methanesulfonate salt that would prevent the conversion of H_2O_2 reaction to OH^* and OH^- by the Fenton reaction. If the latter reaction were important to Lzm-S's vasodilatory effect, then we would have expected to find that the vasodilation by Lzm-S would be relatively inhibited. Wentworth et al also proposed that proteins could also generate OH^* through a series of reactions that involved generation of trihydroxide radical resulting in the formation of $^3\text{O}_2$ and OH^* and that this reaction may not require iron (35). Such a generation of OH^* would be consistent with the pathway leading to the vasodilation caused by Lzm-S in the present study.

Although future studies are required to further elucidate the mechanism by which Lzm-S generates reactive oxygen species, this does not negate the significant findings of the present study showing that Lzm-S causes vasodilation by activation of the cGMP pathway, and that the initiating event appears to be the generation of H_2O_2 and OH^* . Thus, in addition to its myocardial depressant activity, these results suggest

that Lzm-S may contribute to the low systemic vascular resistance found in septic shock by a novel mechanism that involves the generation of reactive oxygen species. Burgess et al (4) showed that blood Lzm-S concentrations increase in patients with abdominal sepsis, so that we think that the findings in our canine models are relevant to what occurs in the human condition. Furthermore, in our previous studies, we showed that competitive inhibitors of Lzm-S's catalytic site, particularly chitotriose and chitobiose, were useful in the treatment of myocardial depression in canine models of septic shock (21), but in the present study, these agents could not prevent Lzm-S-induced vasorelaxation. A therapy that also targets against Lzm-S's vasodilator effect may therefore be of further benefit in the treatment of cardiovascular collapse in septic shock.

Thus, the inhibitors capable of reducing vasodilation caused by lysozyme may be administered to an individual in need of such treatment, for example, an individual undergoing vasodilation during sepsis or experiencing vasodilatory response due to other conditions or circumstances promoting production of H_2O_2 by lysozyme.

As will be appreciated by one of skill in the art, 'reducing' refers to a reduction in vasodilation compared to an untreated or mock treated control of similar condition. It is important to note that the control does not necessarily need to be repeated each time.

In one aspect of the invention, there is provided a method of reducing a vasodilatory response comprising administering to an individual in need of such treatment an effective amount of a guanylate cyclase pathway inhibitor or an H_2O_2 reducing agent.

As will be appreciated by one of skill in the art, 'an effective amount' refers to an amount that is sufficient to reduce vasodilation as discussed herein and will of course depend on the age, weight and condition of the individual in need of such treatment.

As discussed below, the guanylate cyclase pathway inhibitor may be for example but by no means limited to methylene blue, ODQ, LY83583, and a protein kinase G inhibitor.

The H₂O₂ reducing agent refers to an agent capable of reducing the levels of H₂O₂ and may be for example although by no means limited to *Aspergillus niger* catalase and hydroxyl scavengers.

The hydroxyl scavengers may be for example but are by no means limited to
5 hydroquinone or mannitol.

RESULTS

An example of the vasodilatory effect of canine Lzm-S is shown in Panel A in Figure 1, while that of human Lzm-S is depicted in Panel B. In Figure 2 (Panel A), the
10 mean changes in contraction over the course of the study are shown. At a concentration of 1.2×10^{-6} mol/L, canine Lzm-S caused a decrease in contraction of approximately 50% at 30 minutes as compared with at pre-Lzm-S value, while there were smaller effects at the lesser concentrations. For the human Lzm-S experiment, phenylephrine also caused a similar increase in contraction among the three groups.
15 As shown in Figure 2 (Panel B), at a concentration of 3.3×10^{-7} mol/L, Lzm-S resulted in a minimal vasodilatory effect. For the higher concentration, however, there was a marked reduction in contraction in which Lzm-S nearly eliminated the increase in force induced by phenylephrine. Since human Lzm-S appeared to produce a large degree of vasorelaxation at 6.7×10^{-7} mol/L, this concentration of Lzm-S was used in
20 the experiments described below.

Pretreatment with L-NMMA, mechanical removal of the endothelium, indomethacin, and inhibitors of Lzm-S's catalytic site did not prevent vasodilation.

It was initially determined whether pretreatment with L-NMMA would prevent
25 the vasodilatory effect of human Lzm-S. An example from these experiments is shown in Figure 3, Panel A. In the L-NMMA treated group, phenylephrine caused a mean increase in resting force that averaged 6.2 ± 2.4 grams, and contraction increased further to 8.1 ± 3.5 grams with L-NMMA treatment. In the non-L-NMMA treated group, phenylephrine caused a mean increase in contraction from resting
30 force of 7.1 ± 2.1 grams, and contraction remained stable at 6.7 ± 2.1 over the 30

minutes prior to Lzm-S administration. Despite the additional constriction observed with L-NMMA, Lzm-S produced a marked degree of relaxation that occurred to a similar extent in both the L-NMMA treated and non-treated groups. In the L-NMMA treated group (n=8) [relative to the value obtained post-L-NMMA treatment], Lzm-S caused a decrease in contraction to $37 \pm 22\%^*$ at 5 minutes, $13 \pm 16\%^*$ at 15 minutes, and $-3 \pm 1\%^*$ at 30 minutes (the minus value indicates that contraction was below resting force) ($*P < .01$ vs pre-Lzm-S plateau). In the non-L-NMMA treated group (n=12), relative to the value found pre-Lzm-S treatment, Lzm-S caused a decrease in contraction to $23 \pm 24\%^*$ at 5 minutes, $8 \pm 27\%$ at 15* minutes, and $2 \pm 34\%^*$ at 30 minutes ($*P < .01$ vs pre-Lzm-S plateau) [note, also, that in the statistical analysis, the results were compared in which force (in grams) rather than percentage declines were used in the analysis].

In the experiments performed in which the endothelium was mechanically removed, there was no inhibitory effect on Lzm-S's vasodilatory response. An example is shown in Figure 3, Panel B. There was no difference in the phenylephrine response in the endothelial denuded and endothelial intact preparations (7.1 ± 0.5 vs 6.7 ± 0.7 grams). In the denuded preparation (n=4), the mean results showed that Lzm-S caused a decrease in contraction to $79 \pm 18\%$ of the phenylephrine plateau at 5 minutes, $58 \pm 21\%^*$ at 15 minutes, and $54 \pm 20\%^*$ at 30 minutes ($*P < .05$ vs phenylephrine plateau). In the intact preparation (n=4), Lzm-S caused a decrease in contraction to $64 \pm 25\%$ of the phenylephrine plateau at 5 minutes, $53 \pm 33\%^*$ at 15 minutes, and $76 \pm 21\%$ at 30 minutes ($*P < .05$ vs phenylephrine plateau).

A similar lack of an effect was observed with indomethacin 10^{-6} mol/L treatment. In the indomethacin treated group, the mean increase in contraction from resting force with phenylephrine averaged 7.5 ± 1.4 grams and force changed little over 30 minutes to 5.6 ± 0.5 grams when indomethacin was added. In the non-indomethacin time control group, the mean increase in force from resting tension with phenylephrine averaged 6.9 ± 2.7 grams and remained stable over 30 minutes at 6.1 ± 1.8 grams. Despite indomethacin treatment, Lzm-S still produced marked vasodilation that occurred to a similar extent in both the indomethacin treated and

non-treated groups. In the indomethacin treated group (n=5), relative to the post-indomethacin plateau, Lzm-S caused a decrease in contraction to $79 \pm 11\%^*$ at 5 minutes, $57 \pm 24\%^*$ at 15 minutes, and $39 \pm 25\%^*$ at 30 minutes (*P<.01 vs pre-Lzm-S plateau). In the non-indomethacin treated group (n=4), relative to the pre-Lzm-S contraction, contraction decreased to $71 \pm 16\%$ at 5 minutes, $45 \pm 19\%^*$ at 15 minutes, and $19 \pm 15\%^*$ at 30 minutes (*P<.01 vs pre-Lzm-S plateau).

In addition, it was found that the competitive inhibitors of Lzm-S's catalytic site, namely chitobiose and chitotriose, did not block Lzm-S's vasodilatory response. The mean results are shown in Table 1.

Inhibitors of the cGMP pathway prevented Lzm-S-induced vasorelaxation.

Since generation of cGMP, a known second messenger of vasodilation could be involved in Lzm-S's response, inhibitory compounds of this pathway were tested. These compounds included methylene blue (5.5×10^{-5} mol/L), LY 83583 (10^{-4} mol/L), ODQ (10^{-4} mol/L), and a PKG inhibitor (10^{-4} mol/L). All of these agents were found to inhibit Lzm-S's effect. An example for methylene blue is shown in Figure 4. The mean results of the four inhibitors are shown in Figure 5.

Agents that modify H₂O₂ metabolism also inhibited the vasodilation caused by Lzm-S.

Based on the work of Wolin and colleagues (2, 3, 7), it was determined whether agents known to alter H₂O₂ metabolism might inhibit Lzm-S induced vasorelaxation. The peroxidase metabolizing enzyme, catalase from *Aspergillus niger* nearly completely inhibited the vasodilation due to Lzm-S (see Panels A and B in Figure 6). Ethanol, which inhibits Compound I, was also markedly effective at the various concentrations that were used in prevention of Lzm-S- induced vasodilation (see Figure 7). As shown in this figure, it was found that the higher concentrations of ethanol caused an initial vasoconstriction, but there was little change in force when Lzm-S was added to the preparation. In addition, in ethanol treated control groups, in which HEPES buffer rather than Lzm-S was added to the preparation, there was also no change in force with the buffer solution was added. In these control experiments, ethanol at the highest concentration (2×10^{-7} mol/L) caused a slight increase in

contraction as compared with the phenylephrine contraction alone (8.2 ± 2.6 grams to 10 ± 2.6 grams; $P < .05$ vs baseline), but force did not change further when HEPES buffer was added (10.7 ± 2.8 grams at 5 minutes, 11 ± 2.8 grams at 15 minutes, and 11 ± 2.6 grams at 30 minutes).

Moreover, an unrelated alcohol, n-butanol, had no inhibitory effect on Lzm-S induced vasorelaxation. In contrast to ethanol, which caused an initial vasoconstriction, n-butanol caused an initial vasorelaxation in which force decrease to $61 \pm 17\%$ of the phenylephrine- plateau response after this alcohol was added ($P < .001$ vs plateau response). The addition of Lzm-S caused further vasodilation to $84 \pm 9\%$ of the butanol -plateau at 5 minutes, to $62 \pm 21\%^*$ at 15 minutes, and to $23 \pm 27\%^*$ at 30 minutes ($*P < .05$ vs plateau response). These findings were not different from those observed in a Lzm-S treated group in which n-butanol treatment was not administered.

The effect of H_2O_2 on producing vasodilation is shown in Figure 8. There was a biphasic time-effect on force at the different doses used. At concentrations between 10^{-3} to 10^{-1} mol/L, the initial response was one of vasoconstriction that was rapidly followed by vasodilation. At a concentration of 10^{-4} mol/L, there was progressive vasorelaxation over time. At a concentration of 10^{-5} mol/L, there was a minimal effect of H_2O_2 on precontracted force.

To determine whether the hydroxyl radical was involved in Lzm-S's effect, it was assessed whether the hydroxyl radical (OH^*) scavengers, mannitol and hydroxyquinone (22), prevented Lzm-S induced vasorelaxation (see Figure 9). Both treatments had an inhibitory effect.

In screening experiments (data not shown), neither DECTA (10^{-2} mol/L), deferoxamine methanesulfonate salt (10^{-2} mol/L), nor the reducing agents N-acetylcysteine (10^{-3} mol/L), ascorbic acid (10^{-4} mol/L), and reduced-glutathione (10^{-3} mol/L) were found to inhibit Lzm-S- induced vasodilation, so that additional experiments were not performed.

METHODS

Carotid artery ring preparation

Internal carotid arteries (4 cm length) were removed from mongrel dogs (15 to 25 kg) previously anesthetized with pentobarbital (45 mg/kg) (23,36). The arteries were placed in cold HEPES-buffered physiological solution (in mmol/L: 118 NaCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.4 KH₂PO₄, 4.9 KCl, 25 HEPES and 11 glucose) bubbled with a 100 %O₂. The arteries were dissected free from the surrounding tissues to obtain ≈ 4mm diam rings. The rings were suspended in a 10 ml organ bath set at 37° with a pH of 7.35 by means of 2 stain-less steel triangles in which the ring is stretched at optimal length (to ≈ 4 g). The rationale for using the carotid vasculature rather than other systemic vessels was that more vasculature rings could be obtained from the carotid artery.

In all of the protocols described below, the carotid rings were preconstricted with phenylephrine (10⁻⁵ mol/L) (23). Measurements were determined at approximately 20 minutes post phenylephrine instillation when a stable plateau had been reached. After Lzm-S or placebo treatment was added to the preparation, measurements were obtained at 5 minutes, 15 minutes, and 30 minutes post instillation.

In initial experiments, it was determined the degree to which canine Lzm-S produced vasodilation in the precontracted carotid artery preparation. The plasma concentration of Lzm-S previously determined in our sepsis model is approximately 10⁻⁹ to 10⁻⁶ mol/L (20). The three concentrations of canine Lzm-S used in individual experiments were 6 x 10⁻⁷ mol/L, 9 x 10⁻⁷ mol/L, and 1.2 x 10⁻⁶ mol/L, respectively. The results obtained with Lzm-S were compared with those determined over an identical interval in which an equivalent amount of buffer solution was placed into the tissue bath. Canine Lzm-S was purified as previously described from the spleens of non-septic dogs by ARVYS Proteins, Inc (Stamford, CT) (20). The turbidimetric method of Shugar was used to confirm the presence of Lzm-S's enzymatic activity in the preparation (29). The purity of the preparation was determined by the finding of a single molecule species on MS/MS mass spectroscopy that was performed by WM

Keck Foundation Biotechnology Resource Laboratory, Yale University (New Haven, CT).

In addition, the effect of human Lzm-S on producing vasodilation in the precontracted carotid artery ring preparation was examined. The objective was to determine whether human Lzm-S produced changes in vasomotor tone comparable to that found with canine Lzm-S. Human Lzm-S was purchased from EMD Biosciences (San Diego, CA). The two concentrations of human Lzm-S used in individual experiments were 3.3×10^{-7} mol/L and 6.7×10^{-7} mol/L, respectively. Although the degree to which vasodilation might occur was variable among the different batches of lysozyme bought from the same company, it should be noted that same batch was always used in all of the Lzm-S treated muscles in a given study.

To determine whether NO release, the endothelium, or prostanoids contribute to Lzm-S- induced carotid artery vasodilation.

In one set of experiments, it was determined whether the release of NO by the carotid artery endothelium was responsible for the vasodilation caused by Lzm-S. Human Lzm-S was used in all of the experiments that follow, since the human preparation appeared to produce a more consistently potent vasodilatory response as compared with the canine preparation (see Results). In these NO experiments, after contraction was produced by phenylephrine, L-NMMA was added to the tissue bath at 10^{-3} mol/L (AG Scientific, Inc, San Diego, CA) for approximately 30 minutes to allow for maximal inhibitory effect (31). Immediately after instillation of L-NMMA, a vasoconstrictive response was noted that usually stabilized over the 30-minute interval. After stabilization was achieved, human Lzm-S at a concentration of 6.7×10^{-7} mol/L was added to the preparation. Measurements were expressed relative to the steady state contraction determined post- L-NMMA at 5 minutes, 15 minutes, and 30 minutes after Lzm-S instillation. The results were compared between L-NMMA treated and non-treated (HEPES buffer) groups over the aforementioned intervals.

In protocols analogous to those described for the L-NMMA experiments, it was also determined whether pretreatment with indomethacin (10^{-6} mol/L) to prevent prostaglandin release (14) and whether pretreatment with the traditional competitive

inhibitors of Lzm-S [ie N,N'-diacetylchitobiose-chitobiose (10^{-3} mol/L) or N,N',N''-triacetylchiotriose-chitotriose (10^{-3} mol/L)] (Sigma Corp, Oakville, Ont) (21,27) would prevent Lzm-S's vasodilatory effect.

After completion of the above studies, it was examined whether the carotid artery endothelium was necessary for Lzm-S-induced vasorelaxation. In these experiments, the endothelium was mechanically denuded from the carotid ring by means of a cotton-tipped applicator (2, 3, 7, 18). The vasomotor response to carbachol (10^{-3} mol/L) was used to determine whether the endothelium was successfully removed. Carbachol produces vasodilation in the intact preparation, while in the denuded preparation, vasoconstriction is predominantly observed. After denudation of endothelium was confirmed, the ring preparation was washed to remove the excess carbachol. After stabilization of the preparation, phenylephrine was then added to produce contraction. At the three predetermined intervals, the effect of Lzm-S was compared between the endothelial-denuded and intact preparations.

Since L-NMMA, indomethacin, the competitive inhibitors of Lzm-S (ie N,N'-diacetylchitobiose and N,N',N''-triacetylchitotriose), as well as removal of the endothelium did not inhibit Lzm-S induced vasodilation (see Results), subsequent screening experiments were performed to examine whether other pathways may be involved. Among others, these studies included experiments that involved inhibition of the bradykinin β_1 and β_2 receptors by B9340 (11), inhibition of adenylyl cyclase production by SQ 22, 536 (16), inhibition of potassium channels by a high KCl bath (30 mmol), 20-hydroxyeicosatetraenoic acid (30), tetraethylammonium, 4-aminopyridine, charybdotoxin and iberiotoxin (6), inhibition of β receptor activation by propranolol, inhibition of the muscarinic receptor by atropine, and inhibition of the histamine receptor by dipheniramine hydrochloride. Since none of these inhibitors prevented Lzm-S's vasodilatory response, other pathways were considered.

To determine whether activation of soluble form of guanylate cyclase (sGC) to generate cGMP contributes to Lzm-S- induced vasodilation

Since generation of cGMP, a known second messenger of vasodilation could be involved in Lzm-S's response (2,3), inhibitors of the cGMP pathway that included - LY 83583 (6-anilino-5,8-quinolinequinone, 10^{-4} mol/L, EMD, San Diego), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 10^{-4} mol/L, EMD, San Diego CA), methylene
5 blue (5.5×10^{-5} mol/L), and the protein kinase G inhibitor guanosine 3',5' -cyclic monophosphorothioate, β -phenyl-1,N²-etheno-8-bromo-Rp-isomer, sodium salt (EMD, San Diego CA) (10^{-6} mol/L)- were examined (2, 3). In respective experiments, the carotid ring was incubated with one of these agents for approximately 30 minutes prior to phenylephrine induced contraction. After a stable phenylephrine response
10 was achieved, the effect of human Lzm-S (6.7×10^{-7} mol/L) on inhibition of the vasodilatory response was determined at 5, 15, and 30 minutes post-instillation.

To determine whether Lzm-S induced vasodilation may be prevented by a peroxidase metabolizing enzyme and hydroxyl radical scavengers.

In precontracted isolated bovine intrapulmonary arterial rings, Burke and Wolin
15 (2) showed that hydrogen peroxide (H_2O_2) produces a concentration dependent relaxation by a mechanism that is independent of the endothelium. They found that micromolar concentrations of H_2O_2 elicited increases in cGMP concentrations that were associated with decreases in force. This effect could be inhibited by prior treatment with the peroxidase metabolizing enzyme, catalase from *Aspergillus niger*.
20 They further showed that H_2O_2 caused relaxation by interacting with endogenous catalase in which their results were most consistent with the involvement of Compound I, a species of catalase formed during the metabolism of endogenous peroxide, in the mechanism of cGC activation. This laboratory additionally showed that cGMP generation could be inhibited by ethanol that selectively interacts with
25 Compound I (3) (see Discussion). In a rat liver preparation, Mittal and Murad (22) also considered that H_2O_2 could generate cGMP and proposed that hydroxyl radicals were also necessary for this generation to occur.

In our carotid artery preparation, the applicability of H_2O_2 production to Lzm-S-induced relaxation was determined within the framework of the experiments
30 delineated by Wolin and colleagues (2,7). In one set of experiments, it was assessed

whether ethanol (range: 10^{-8} mol/L to 2×10^{-7} mol/L) prevented Lzm-S's vasodilatory effect. The results obtained with ethanol were contrasted to those obtained when another alcohol -n-butanol (10^{-7} mol/L) was administered in order to assess whether this effect was specific for ethanol. It was also determined whether prior incubation of

5 the carotid artery preparation with the peroxidase metabolizing enzyme, catalase from *Aspergillus niger* (10^{-6} mo/L, Sigma Corp, Oakville On) and whether the addition of the hydroxyl radical (OH^*) scavengers (21) mannitol (10^{-3} mol to 10^{-1} mol/L), and hydroxyquinone (10^{-3} mol/L to 10^{-4} mol/L) would inhibit Lzm-S-induced vasorelaxation.

In another experiment, it was examined whether H_2O_2 by itself (range: 10^{-5}

10 mol/L to 10^{-1} mol/L) would produce vasodilation in a manner similar to that found for Lzm-S. Finally, in respective experiments, it was assessed whether Lzm-S-induced vasodilation could be prevented by diethyldithiocarbamic acid (DECTA) which inhibits Zn^{+2} , Cu^{+2} superoxide dismutase (SOD) (7), since superoxide generation could be important in Lzm-S's effect; by the iron chelator deferoxamine methanesulfonate salt

15 (10^{-2} mol/L) (5), which would prevent the conversion of H_2O_2 reaction to OH^* and OH^- (by the Fenton reaction); and by reducing agents that included N-acetylcysteine (10^{-3} mol/L), ascorbic acid (10^{-4} mol/L), and reduced glutathione (10^{-3} mol/L).

Statistics. Differences in vasodilation among groups were determined by a two way (between –within ANOVA) analysis of variance. Student Newman Keuls' multiple comparison test was included to determine statistical differences between

20 treatment groups when the ANOVA was used. In the statistical analysis, moreover, the results were compared in which force (in grams) rather than percentage declines were used in the analysis. In the design of the experiment, of the 8 to 10 rings obtained from a carotid artery of a given dog, each ring was used for a different

25 subset of experiments in a specific study. Results are given as mean ($\pm 1\text{SD}$).

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.

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CLAIMS

1. A method of reducing a vasodilatory response comprising administering to an individual in need of such treatment an effective amount of a guanylate cyclase pathway inhibitor or an H₂O₂ reducing agent.
- 5 2. The method according to claim 1 wherein the guanylate cyclase pathway inhibitor is selected from the group consisting of methylene blue, ODQ, LY83583, and a protein kinase G inhibitor.
3. The method according to claim 1 wherein the H₂O₂ reducing agent is selected from the group consisting of: *Aspergillus niger* catalase and
10 hydroxyl scavengers.
4. The method according to claim 3 wherein the hydroxyl scavengers are hydroquinone or mannitol.

1 / 9

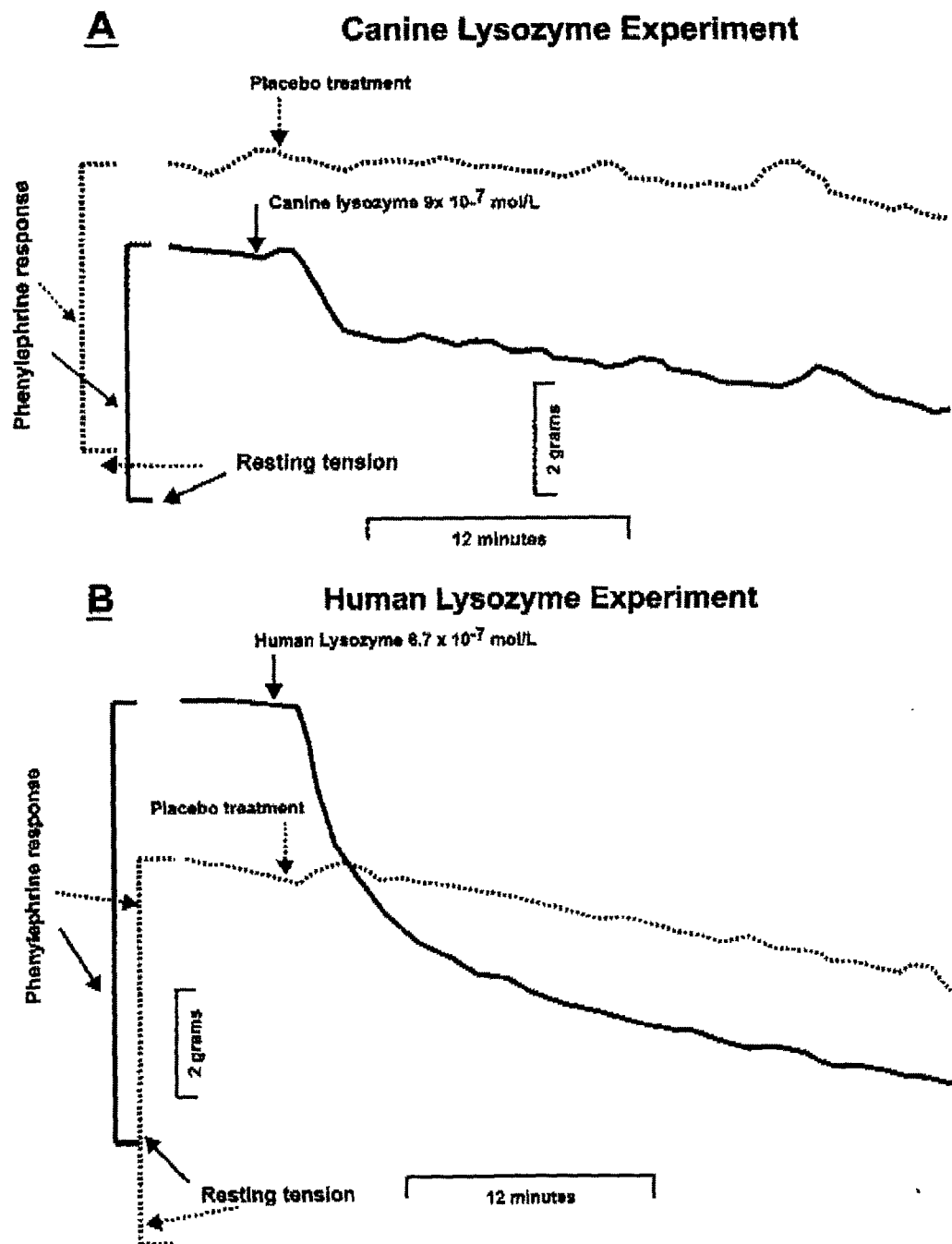


Figure 1

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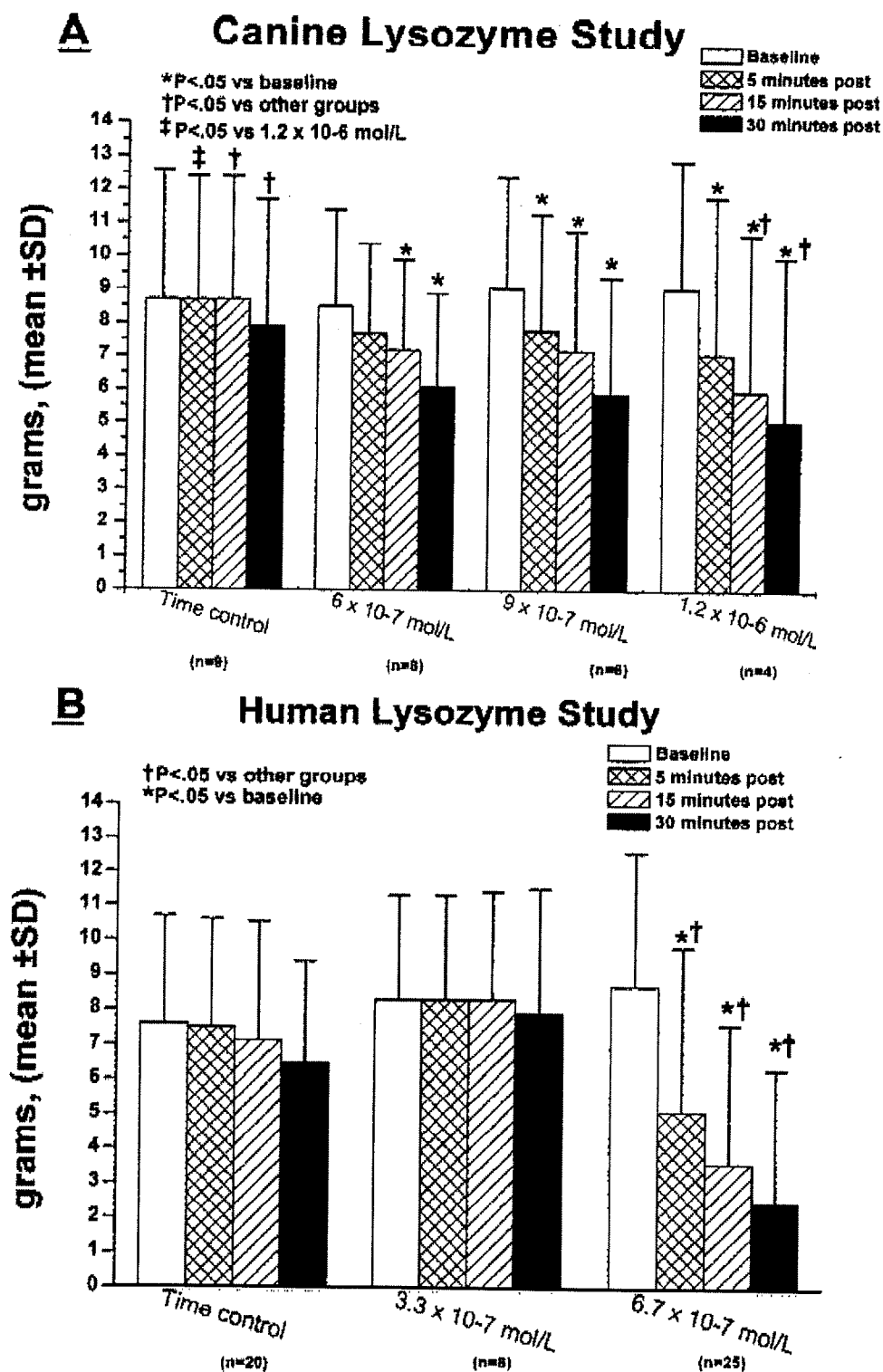


Figure 2

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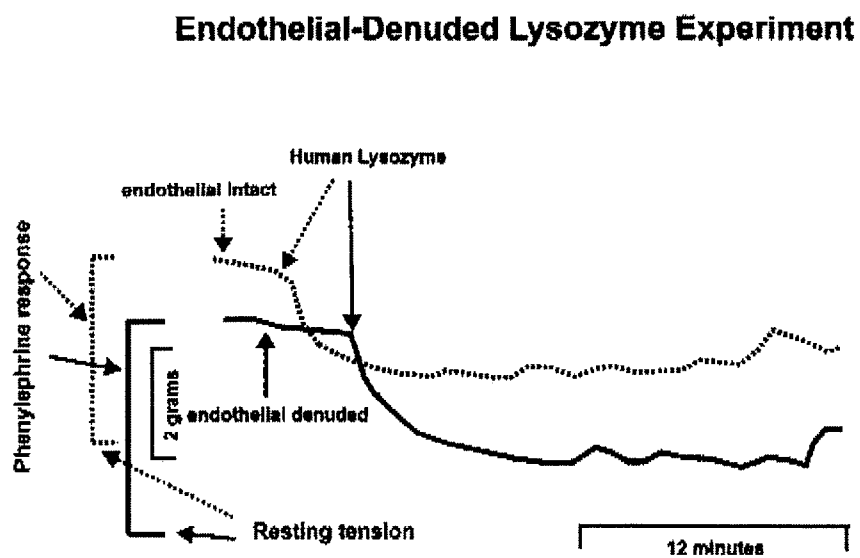
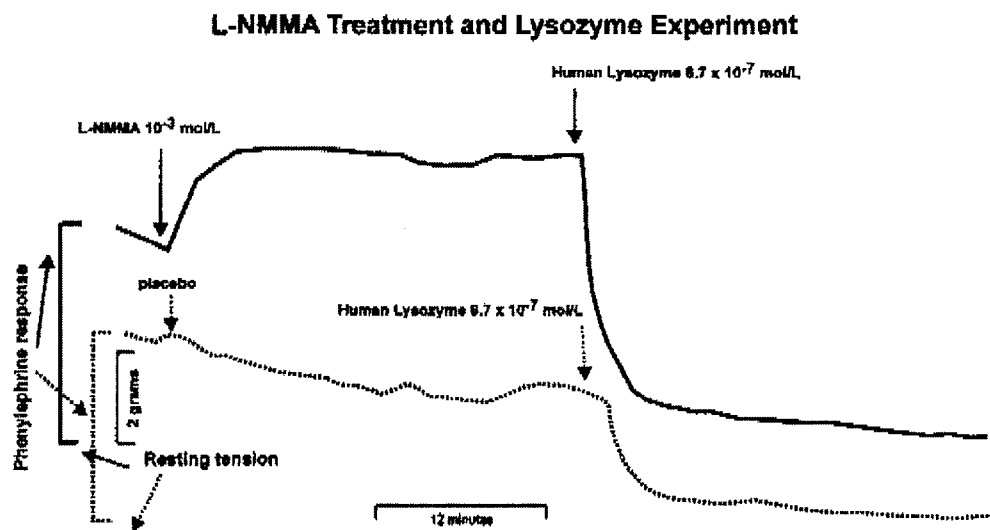


Figure 3

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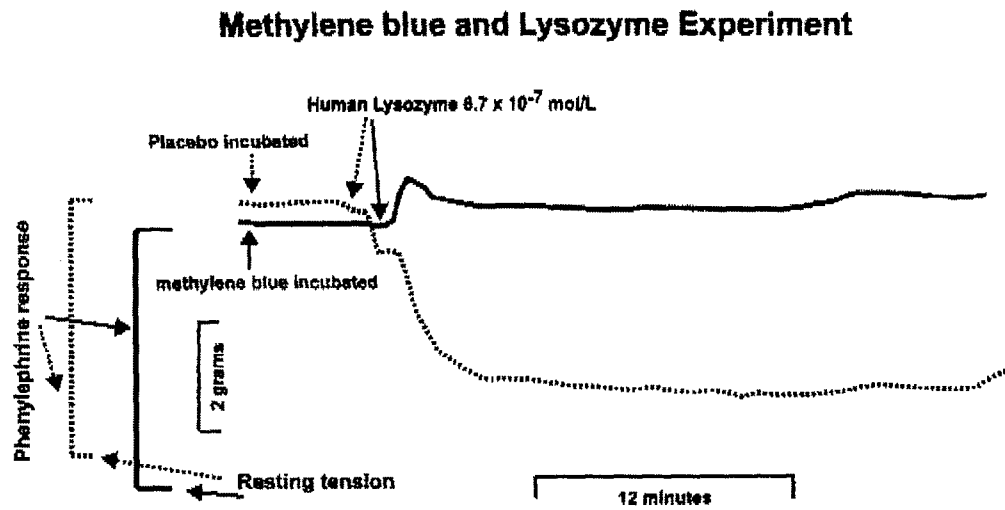


Figure 4

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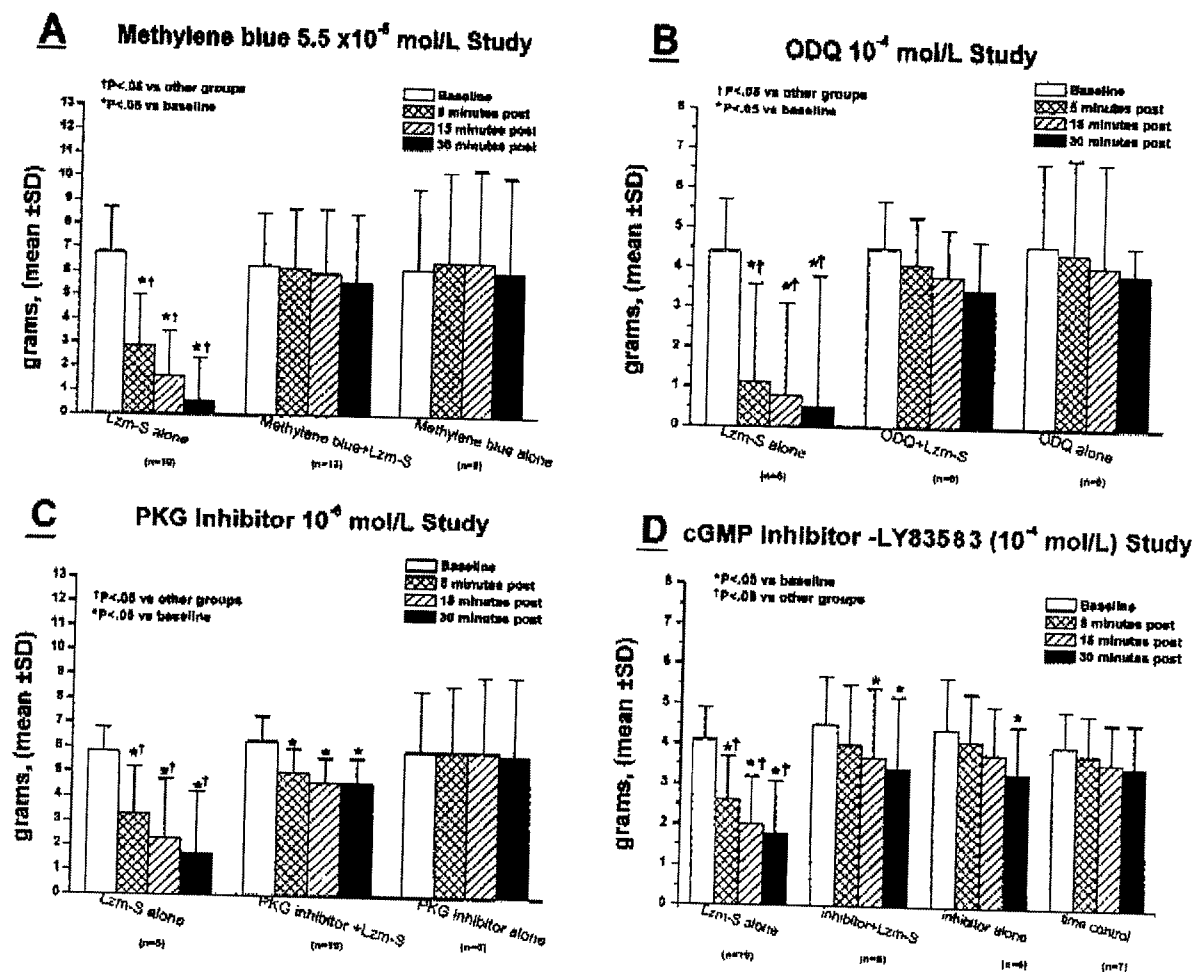


Figure 5

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Peroxidase metabolizing *Aspergillus niger* catalase (10⁻⁶ mol/L) Study

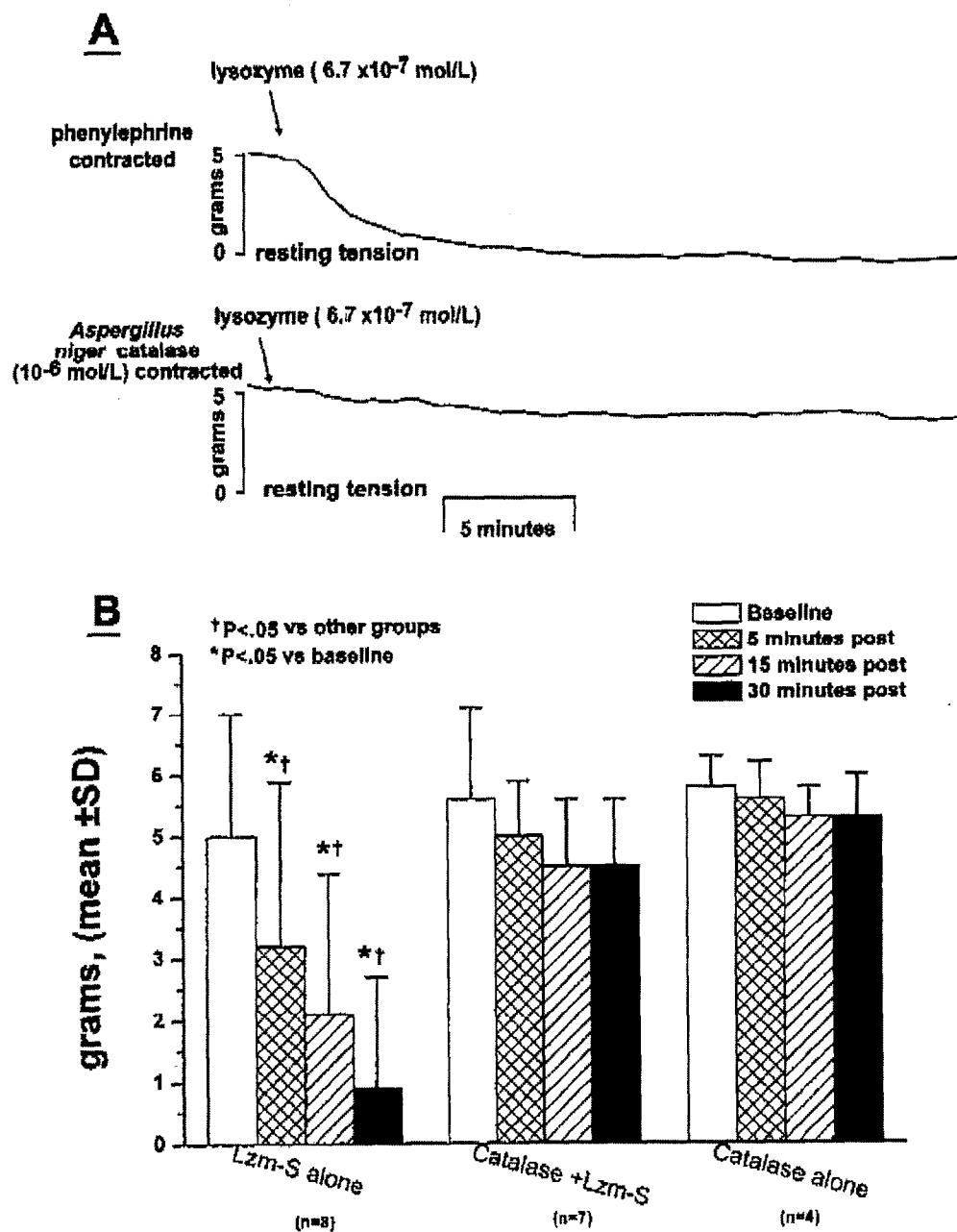


Figure 6

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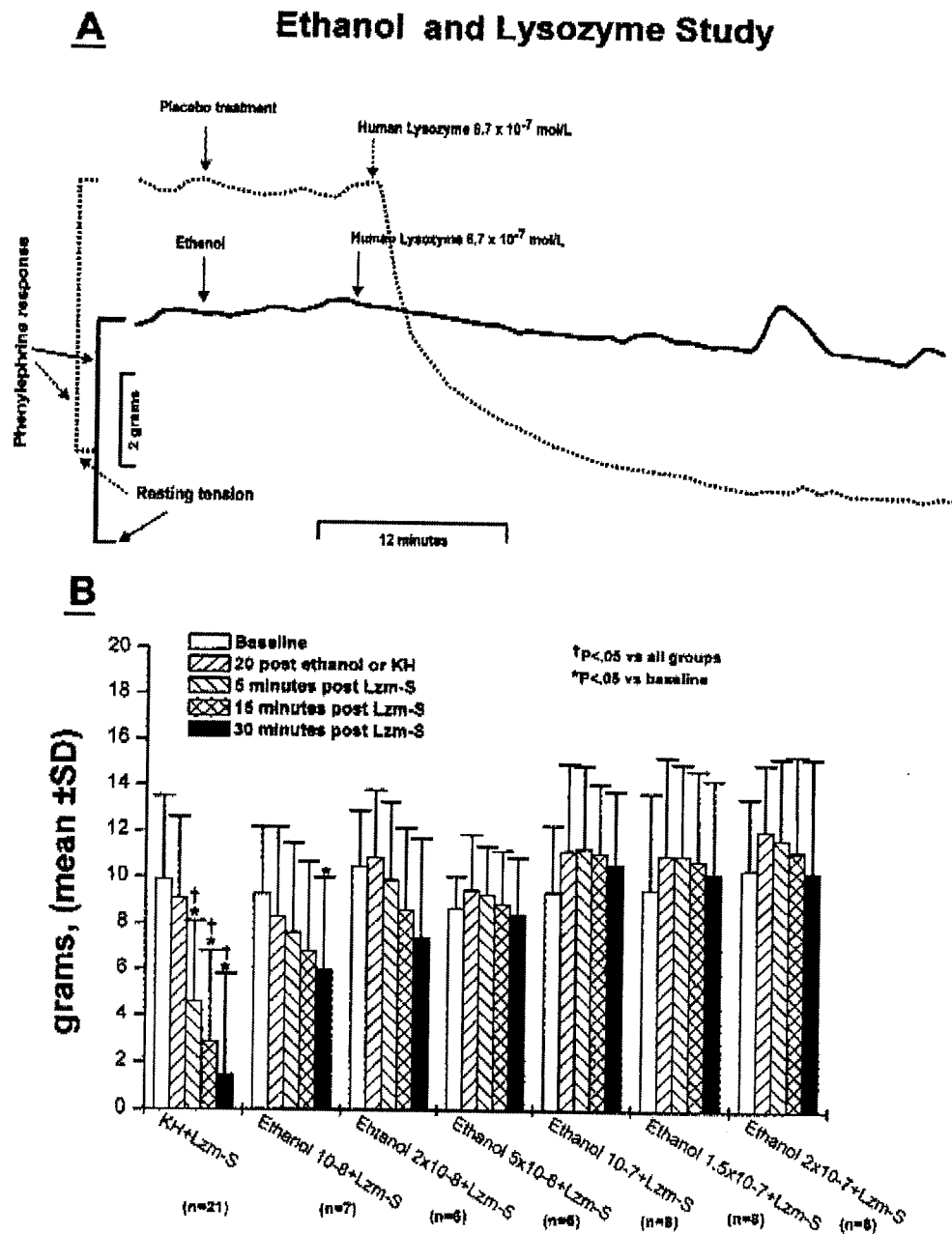


Figure 7

H₂O₂ Study

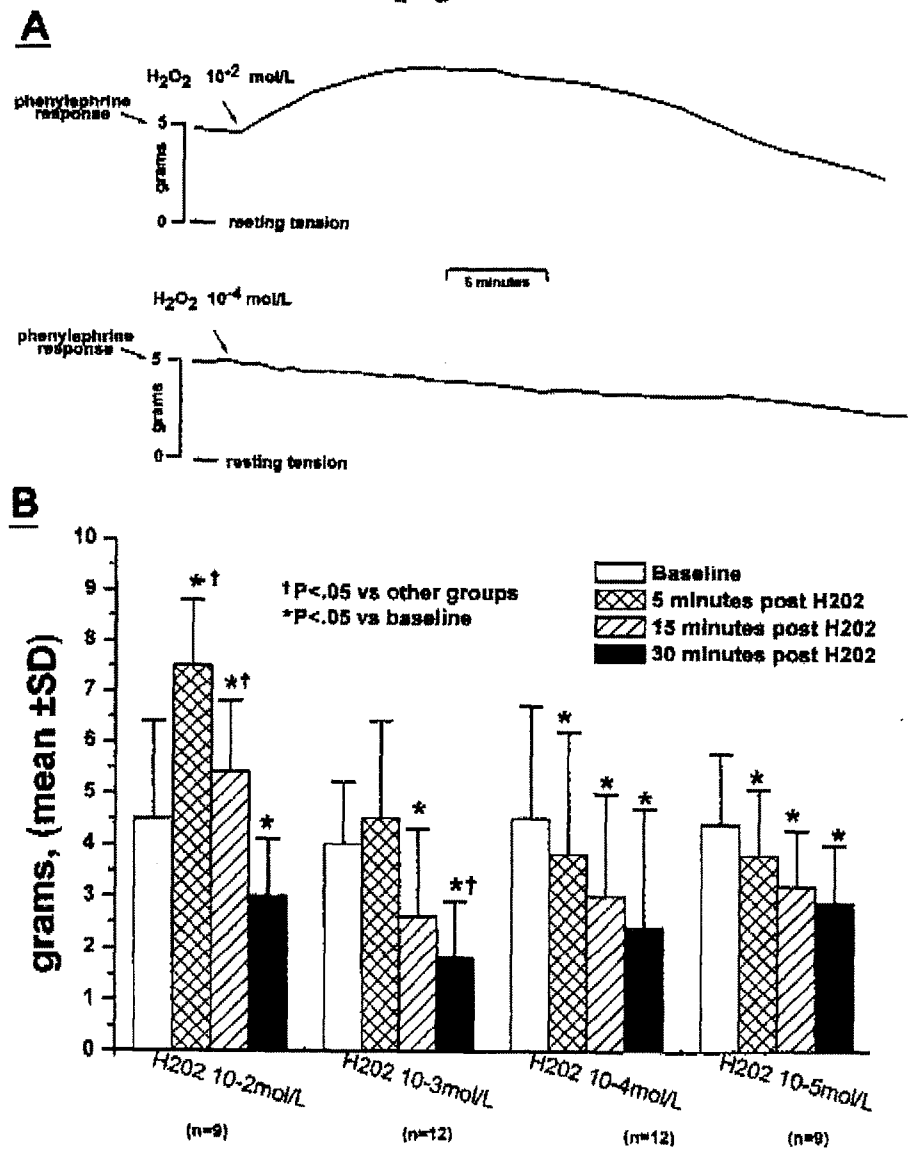


Figure 8

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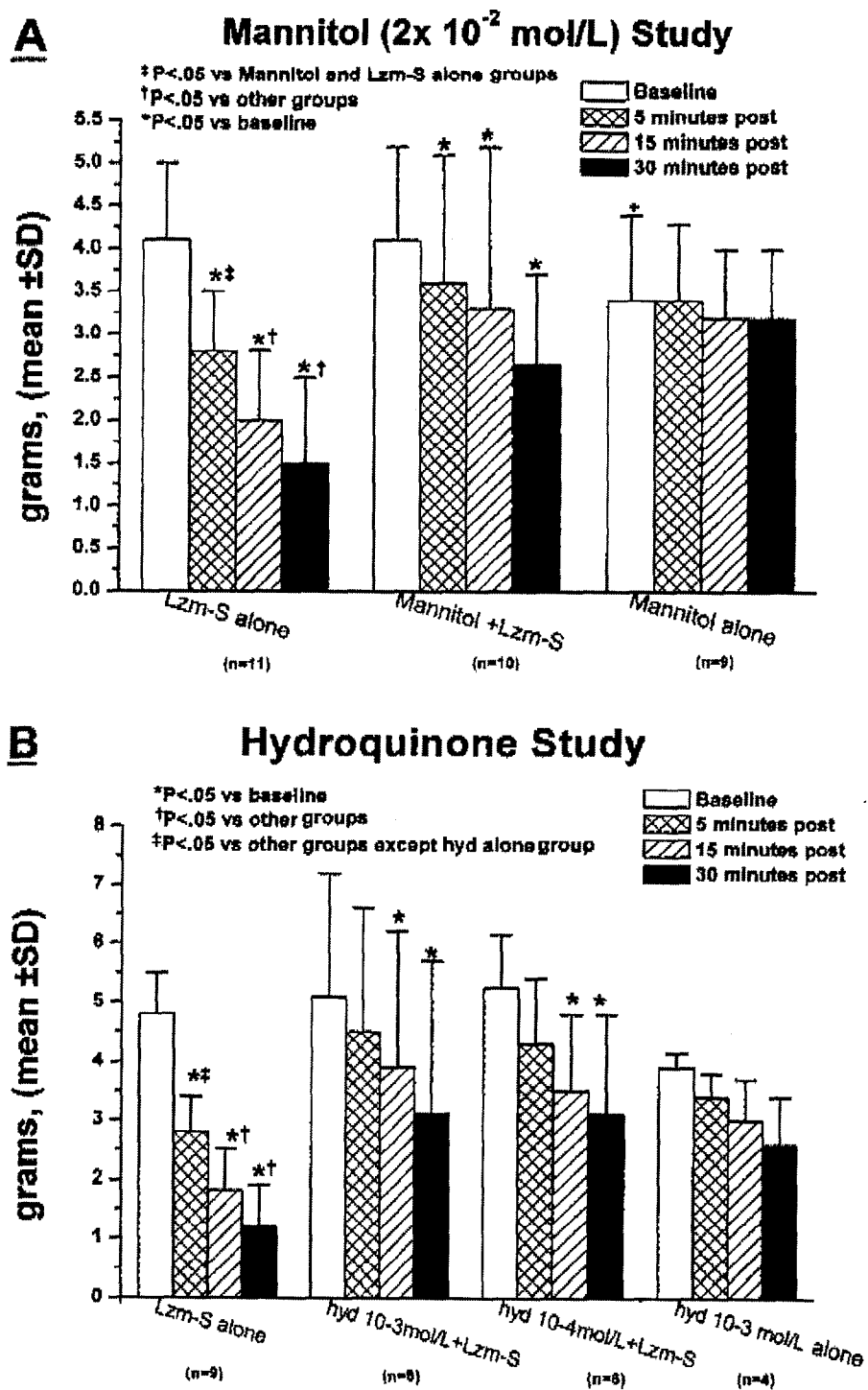


Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001513

A. CLASSIFICATION OF SUBJECT MATTER IPC: A61K 38/44 (2006.01) , A61K 31/047 (2006.01) , A61K 31/05 (2006.01) , A61K 31/47 (2006.01) , A61K 31/4985 (2006.01) , A61K 31/5415 (2006.01) (more IPCs on the last page) According to International Patent Classification (IPC) or to both national classification and IPC																										
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K 38/44, A61K 31/047, A61K 31/05, A61K 31/47, A61K 31/4985, A61K 31/5415 and A61P 9/00 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Canadian Patent Database, PubMed, Delphion, GenomeQuest and Scopus. Keywords: vasodilation, inhibitors, guanylate cyclase pathway, guanylate, H2O2, peroxide, reducing agent, methylene blue, ODQ, Ly83583, protein kinase G inhibitor, aspergillus niger catalase, hydroxy scavengers, hydroquinone and mannitol.																										
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>PARK B. ET AL. The effects of Methylene Blue on hemodynamic parameters and cytokine levels in refractory septic shock. KOREAN J INTERN MED 2005 20: 123 - 128 ISSN: 1226-3303 see abstract and page 24.</td> <td>1 and 2</td> </tr> <tr> <td>X</td> <td>ZINGARELLI B. ET AL. Effects of a novel guanylyl cyclase inhibitor on the vascular actions of nitric oxide and peroxynitrite in immunostimulated smooth muscle cells in endotoxic shock. CRIT CARE MED. 1999 27(9): 1701 - 1707 ISSN: 1530-0293 see abstract and pages 1701, 1702 and 1704.</td> <td>1 and 2</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	PARK B. ET AL. The effects of Methylene Blue on hemodynamic parameters and cytokine levels in refractory septic shock. KOREAN J INTERN MED 2005 20: 123 - 128 ISSN: 1226-3303 see abstract and page 24.	1 and 2	X	ZINGARELLI B. ET AL. Effects of a novel guanylyl cyclase inhibitor on the vascular actions of nitric oxide and peroxynitrite in immunostimulated smooth muscle cells in endotoxic shock. CRIT CARE MED. 1999 27(9): 1701 - 1707 ISSN: 1530-0293 see abstract and pages 1701, 1702 and 1704.	1 and 2															
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex. <table border="1"> <thead> <tr> <th>*</th> <th>Special categories of cited documents :</th> <th>"T"</th> <th>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</th> </tr> </thead> <tbody> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td></td> <td></td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </tbody> </table>			*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E"	earlier application or patent but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family	"O"	document referring to an oral disclosure, use, exhibition or other means			"P"	document published prior to the international filing date but later than the priority date claimed		
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Date of the actual completion of the international search 28 October 2008 (28-10-2008)		Date of mailing of the international search report 20 November 2008 (20-11-2008)																								
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Ken Steinberg 819- 934-7929																								

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001513**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 1 - 4

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 1 - 4 are directed to methods practiced on a human or mammalian body which the International Search Authority is not required to search under PCT Rule 39.1(iv). However, the search has been carried out based on the use of a guanylate cyclase pathway inhibitor or an H₂O₂ reducing agent in a method of reducing a vasodilatory response.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

Group A - Claims 1 (in part) and 2 (wholly) are directed to methods of reducing a vasodilatory response comprising administering to an individual, an effective amount of a guanylate cyclase pathway inhibitor, **wherein each of the guanylate cyclase pathway inhibitors used in said method is viewed to represent a separate invention; and**

Group B - Claims 1 (in part), 3 and 4 are directed to methods of reducing a vasodilatory response comprising administering to an individual, an effective amount of a H₂O₂ reducing agent, **wherein each of the H₂O₂ reducing agents used in said method is viewed to represent a separate invention.**

As methods of reducing a vasodilatory response utilizing guanylate cyclase pathway inhibitors are disclosed in D1 and H₂O₂ reducing agents are disclosed in D6 and D8 are known in the prior art, thus **a single general inventive concept cannot be identified among the**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001513

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BEASLEY D. AND MCGUIGGIN M. Interleukin 1 activates soluble guanylate cyclase in human vascular smooth muscle cells through a novel nitric oxide-independent pathway. J EXP. MED. 1994 179: 71 - 80 ISSN: 0022-1007 see abstract and page 71, 77 and 78	1 and 2
Y	WU J. Y. ET AL. Effect of protein kinase on endothelial cytoskeleton induced by septic shock. (English Abstract) PMID: 12887778 ZHONGHUA WAI KE ZA ZHI 2003 41(3): 193 - 196 ISSN: 0529-5815 Retrieved on 27-10-2008	1 and 2
Y	RASTALDO R. ET AL. Nitric oxide and cardiac function LIFE SCIENCES 16-08-2007 81: 779 - 793 ISSN: 0024-3205 see abstract and page 780	1 and 2
X	BAKER C. H. AND SUTTON E. T. Arteriolar endothelium dependent vasodilation occurs during endotoxin shock.	1, 3 and 4
Y	(Abstract) PMID: 8476088 AM J PHYSIOL 1993 264(4 Pt 2): H1118 - H1123 ISSN: 0002-9513	1 and 3
Y	US5360732 A (BERKA R. M. ET AL.) 1 Nov 1994 (01-11-1994) see abstract and column 1 and 2	1 and 3
X	KRISHNAMURTY V. S. ET AL. Inhibitory effect of hypertonic mannitol on vasoconstrictor and vasodilator responses of isolated coronary arteries. (Abstract) PMID: 104632 AM J PHYSIOL HEART CIRC PHYSIOL 1978 235(6): H728 - H735 ISSN: 0363-6135 Retrieved on 27-10-2008	1, 3 and 4

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2008/001513

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
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A61P 9/00 (2006.01)