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Is HSP70 Involved in Nitric Oxide-Induced Protection of the Heart?

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Summary

It is known that HSP70 plays an important role in the antiischaemic effect of adaptation to stress. The aim of our study was to verify the hypothesis that nitric oxide (NO) may contribute to the activation of HSP70 synthesis and to enhance thereby the resistance of organism to the ischaemic and reperfusion damages. We observed that heat shock potentiated NO production in the heart. NO formation was completely blocked by the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA). L-NNA also significantly attenuated the heat shock-induced accumulation of HSP70 (by 45 % in heart). Both heat shock and NO donor induced time- and concentration-dependent HSP70 synthesis in the culture of human hepatoblastoma cells Hep G2. Prior injection of NO donor (30-100 mg per rat) exerted a dose-dependent protective effect on the isolated heart in ischaemia and reperfusion within 24 hours. We suggest that NO is involved in the activation of HSP70 synthesis which can play an important role in the delayed protective effect of NO donors.

Key words

Heat shock – Electron paramagnetic resonance – Western blot analysis – Nitric oxide – Stress proteins

Introduction

Heat shock protein (HSP70) synthesis increases in various situations, e.g. heat shock (HS), myocardial ischaemia, hypoxia, cooling, etc. (Schlesinger *et al.* 1982, Welch and Suhan 1986). HSP70 plays an important role in the restriction of stress damage (Pelham 1986) and in the development of antiischaemic effect of adaptation to stress (Meerson and Malyshev 1993) and heat shock (Currie *et al.* 1988, Karmazyn *et al.* 1990). However, the question which cell mechanisms are involved in the activation of HSP70 synthesis remains open in many respects. When analyzing this problem, the experimental results have drawn our attention to the fact that the same agents can activate both HSP70 and nitric oxide (NO) synthesis. For instance, lipopolysaccharides, a classical inductor of NO synthesis, also activate HSP70 synthesis (Zhang *et al.* 1994). On the other hand, heat shock, which is a conventional method of inducing HSP synthesis (Schlesinger *et al.* 1982, Welch and Suhan 1986, Tomasovic 1989), is accompanied by an increase in blood NO-haeme (Hall *et al.* 1994). Our own experiments have demonstrated that adaptation of the organism to stress both activates HSP70 synthesis (Meerson and Malyshev 1993) and potentiates NO production in the same organs (Meerson *et al.* 1994). Taken together, these data suggest that NO may contribute to the activation of HSP70 synthesis and could enhance thereby the organism's resistance to ischaemic and reperfusion damages. The aim of our study was to verify this hypothesis. We studied 1) the effect of HS on the NO production in rat hearts and in cultured cells, 2) the effect of a NO synthase inhibitor on the HS-induced accumulation of HSP70, 3) the effect of a NO donor on HSP70 accumulation in

cultured cells, and 4) the effect of the NO donor on heart resistance to ischaemia and reperfusion.

Methods

Experiments were carried out on Wistar male rats weighing 250–300 g.

Heat shock. HS was produced by heating of conscious animals in a thermostat to the core temperature of 42 °C. After that the heating was continued for additional 15 min (Currie *et al.* 1988). The total duration of heating did not exceed 30 min.

Measurement of NO content. To estimate the amount of NO produced in rat tissues we used the capacity of NO to form ferrous diethyldithiocarbamate (Sigma, USA) complexes and thus to form paramagnetic mononitrosyl iron compounds. This method has been described in detail elsewhere (Vanin *et al.* 1984, Mulsch *et al.* 1992). The EPR signal from the samples was recorded on an EPR-radiospectrometer Radiopan (Poland) at 77 °K, with field modulation amplitude 0.5 mT and wave power 10 mW. Another group of rats received an additional injection of a NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA) (Merck, Germany) (20 mg/100 g body weight, i.p.) immediately after the completion of HS. The rats were decapitated 1, 4 and 24 h after HS.

Electrophoresis and immunoblotting. HSP70 was measured in the cytosolic fraction. The cardiac tissue was ground and placed into a hypotonic buffer (10 mM Tris, 10 mM KCl, pH 7.4) for 10 min at 4 °C. The tissue was then homogenized in the same solution at the buffer:tissue ratio 5:1 (w/w). The homogenate obtained was filtered through eight gauze layers and centrifuged at 12 000 x *g* and 4 °C for 10 min. The supernatant containing cytosolic proteins was taken for electrophoresis and blotting. Electrophoresis was performed according to Laemmli (1970). Proteins were separated in 7 % PAAG and transferred from PAAG to a nitrocellulose membrane according to Towbin (1979). Western blots were successively incubated in the presence of monoclonal antibodies against HSP70 (Amersham, United Kingdom). After washing, the blots were incubated in the presence of [^{125}I]-labelled antimurine IgG (Sigma). HSP70 were detected autoradiographically.

Isolated heart experiments. Animals were heparinized (2000 U/kg i.p.) and anaesthetized with Nembutal (50 mg/kg i.p.). Then the hearts were rapidly excised and placed into a Langendorff perfusion system containing a standard Krebs-Henseleit solution. The mechanical activity of the isolated heart was recorded with a Nihon Kohden (Japan) TD-112S isotonic transducer (Gilst and Koomen 1987). The models of ischaemic and reperfusion damage were reproduced using the method of Hearse *et al.* (1973). The coronary flow was completely interrupted for 15 min, after which perfusion was switched on and the observation continued for 15 min of reperfusion. The severity of reperfusion damage to the isolated heart was evaluated by the decline in contraction amplitude, contracture and arrhythmia. Contraction amplitude was expressed in mm of the absolute change in apicobasal length of the heart (Gilst and Koomen 1987). The cardiac rhythm disorders were recorded by ECG. Dinitrosyl iron complex (DNIC 1:2) was used as the NO donor (Vedernicov *et al.* 1992). DNIC was injected intravenously in doses of 30, 60 and 100 μg per rat. Experiments on the isolated heart were started 24 h after the DNIC injection.

Cell culture. Experiments were carried out using a human hepatoblastoma cell line, Hep G2. Hep G2 cells were grown on 35 mm plastic dishes. The cells were cultured in Eagle's modified minimum essential medium (EMEM) supplemented with 1 % non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, containing 10 % foetal bovine serum (FBS) and incubated in temperature-controlled, humidified incubators (5 % CO_2). The cells at near confluence were subjected to HS by the addition of a fresh medium prewarmed to 42 °C and subsequent incubation at this temperature. The culture medium was removed after 2 h, the cells were washed twice with EMEM and incubated for one hour at 37 °C. At the end of incubation, the cells were washed with cold 1 mM phosphate buffered saline (PBS), pH 7.4, and prepared for the EPR-assay. The DNIC solution was added to the culture medium at the final concentrations of 20 or 100 mM. The cells were incubated with DNIC. After 15 min, 4 h or 24 h the cells and medium were EPR-assayed.

After addition of DNIC, cells were incubated for 4, 8, 16 or 24 h and, at the end of incubation, the cells were washed three times with PBS. Cell pellets were suspended at a concentration of $2x10^7$ cells/ml in 100 ml lysis buffer containing 1.0 % Nonidet P-40, 150 mM NlCL, 10 mM EDTA, 100 mM Tris-HCl at pH 8.0 for 30 min at 4 °C. The insoluble debris was removed by microcentrifugation at 18 000 x *g* for 10 min at 4 °C. Proteins were separated by electrophoresis according to Laemmli (1970) and transferred from PAAG to a nitrocellulose membrane by electroelution according to Towbin (1979). Western blots were successively incubated in the presence of monoclonal anti-72kD heat shock protein (Amersham, United Kingdom). Resulting immune complexes were reacted with horseradish peroxidase-conjugated antimurine Ig (Amersham, United Kingdom). Finally, labelled antigen bands were detected by diaminobenzidine staining.

The results were statistically evaluated by the unpaired Student's t-test and were presented as means ± S.E.M.

Results

HS increases NO production in the rat heart and inhibition of NO synthase reduces HSP70 synthesis after HS

Figure 1 demonstrates that HS sharply increased the NO production in the hearts. The rate of NO accumulation was maximal 1 h after the end of exposure. After 4 h, NO generation began to decline. At 24 h, it did not differ significantly from the initial level. In animals treated with L-NNA, the inhibitor of NO synthase, NO was not detected in any of the organs studied both in control and 1, 4 or 24 h after HS. Twenty-four hours after HS, the HSP70 accumulation was observed in the heart. L-NNA decreased the HS-induced HSP70 accumulation by 45 %.

The results support our hypothesis that one of the mechanisms of the HS-induced increase in HSP70 synthesis is related to the increased NO production.

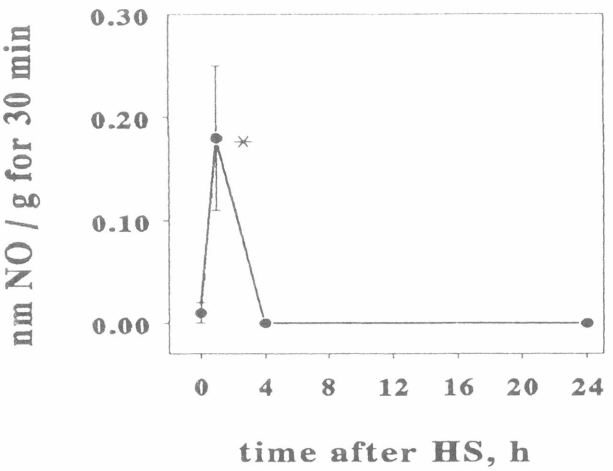

Fig. 1
*Time course of NO production in the rat heart after heat shock. Abscissa: time after heat shock. Ordinate: the rate of NO generation (nM NO/g wet tissue for 30 min). * Significant differences from the initial value, $p<0.05$. Arrow indicates induction of heat shock that lasted 15 min. Five animals were studied in each series.*

HS increases the NO generation, and the NO donor induces synthesis of HSP70 in cell culture

Heating of cultured cells for 2 h at 42 °C resulted in the appearance of endogenous DNIC 1:20 in a concentration 2 nmol/10^6 cells 1 h following HS. This indicated that HS potentiated the production of NO which then formed DNIC with endogenous iron and thiol-containing ligands.

Incubation of hepatocytes with DNIC 1:2 in the concentration of 100 mM resulted in the appearance of DNIC 1:20 derived from DNIC 1:2 in the cells. The intracellular concentration of DNIC 1:20 was 5 nmol/10^6 cells after 15 min of incubation. Detection of the EPR signal from DNIC 1:20 in cells after the contact of cells with exogenous DNIC 1:2 ensures that this NO donor has really entered the cells. Both HS and exogenous DNIC 1:2 administration induced synthesis of HSP70 (Fig. 2). The DNIC-induced HSP70 synthesis was time- and concentration-dependent.

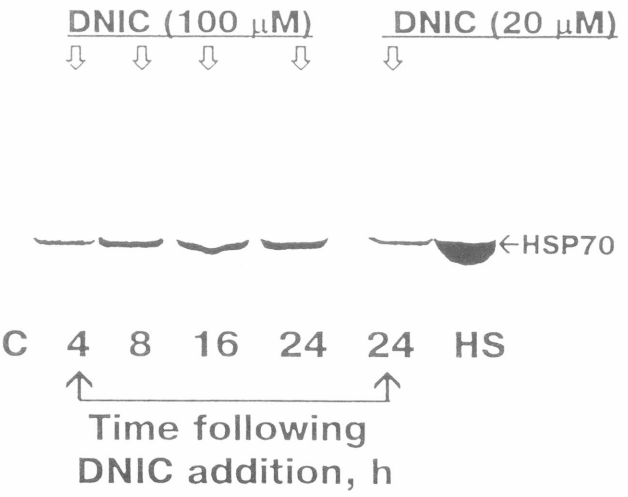

Fig. 2
The effect of NO donor (DNIC 1:2) on HSP70 accumulation in Hep G2 cells.

DNIC enhances the resistance of the isolated heart to reperfusion

Curves in Figure 3 demonstrate the response to total ischaemia and reperfusion of the hearts from control rats and from rats which had received an injection of the NO donor. The initial amplitude of contraction was approximately the same in all the experimental groups. Ischaemia induced a pronounced depression of contraction amplitude in all four groups. In reperfusion, the contraction amplitude of control hearts was 8% of the initial value. In addition, reperfusion induced cardiac fibrillations and arrest in 80 % of control hearts. It follows from Figure 3 that prior injection of 30 μg DNIC did not change the initial contraction amplitude. In ischaemia and reperfusion the depression of contraction amplitude of these hearts was the same as in control. The prior administration of 30 μg DNIC completely prevented reperfusion fibrillations and the arrest of hearts (Fig. 3). When the NO donor was injected in the dose of 60 μg, its protective effect was evident as a significant decrease in the depression of contraction amplitude and in the complete absence of cardiac arrhythmias and arrest during reperfusion (Fig. 3). By the 15th min of reperfusion, for instance, the hearts from control

animals contracted with the amplitude of 0.15±0.12 mm. The contraction amplitude of hearts from rats which had received 60 μg DNIC was 5 times higher than in the controls (Fig. 3). The dose of 100 μg DNIC exerted no protective effect. Moreover the initial amplitude of contraction was somewhat diminished in comparison with control hearts. This may indicate a toxic effect of NO.

Thus DNIC exerts a dose-dependent protective effect and enhances the resistance of the isolated heart to disorders of contractile function and arrhythmias after reperfusion.

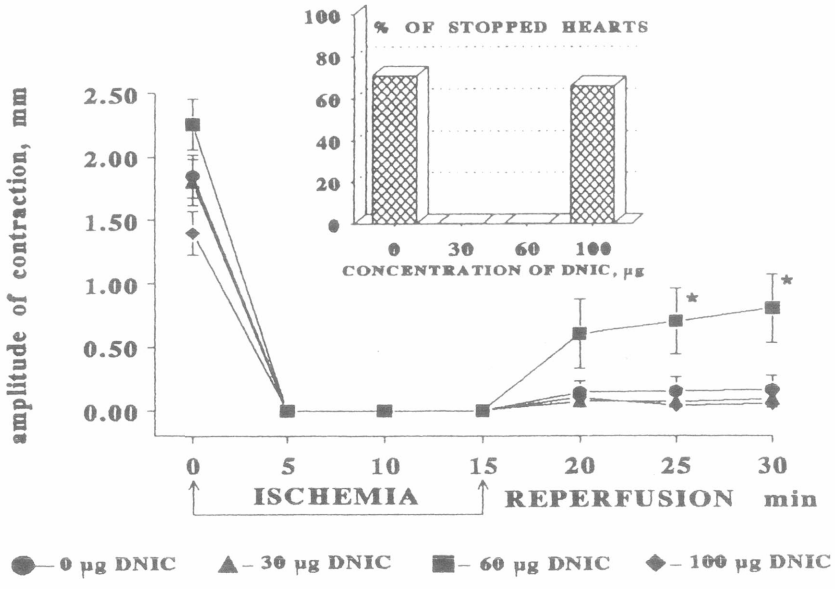

Fig. 3
*The effect of NO donor on the resistance of the isolated heart to ischaemic and reperfusion injuries. Abscissa: time of experiment in min; ordinate: contraction amplitude in mm of apicobasal shortening of the heart. Columns in the insert show the percentage of arrested hearts due to reperfusion-induced fibrillations. * Significant differences from the control, $p<0.05$.*

Discussion

It is commonly accepted that NO is a key molecule in the regulation of various physiological processes including the regulation of immune, nervous and cardiovascular systems (Moncada 1994). Our results supplement this picture: NO can operate as a factor initiating synthesis of HSP70 and thereby enhance myocardial resistance to damage. It seems appropriate to discuss the mechanism of NO-induced activation of HSP70 synthesis. We suggest that the mechanism may be as follows (Fig. 4).

It is known that, under basal conditions, the HSP70 transcription factor (HSF) is inactive. Stress factors induce trimerization of HSF. As a result, trimeric HSF enters the nucleus and activates transcription of HSP70 genes. It is quite possible that NO, specifically its ionized form, nitrozonium ion NO^+, catalyzes the trimerization process and accelerates the formation of disulfide bonds between HSF molecules. Based on current knowledge (Stamler 1994), the reaction appears as follows: NO^+ interacts with the SH-groups of HSF to form an intermediate S-nitrosothiol. Degradation of the latter with formation of highly reactive thiyl radicals results in formation of disulfide bonds between HSF molecules. DNIC can serve as a source of NO^+. In contrast to neutral NO, NO^+ readily nitrosylates various groups including thiols.

The antiischaemic cardioprotective effect of NO donors has recently been demonstrated (Cooke and Tsao 1993). This protective effect developed either immediately after NO donor administration or during NO donor infusion over the entire period of ischaemia and reperfusion. This effect was suggested to be primarily due to the NO ability to suppress the neutrophil adherence to the endothelium and also to the reduced myocardial oxygen demand during ischaemia and reperfusion (largely due to reduction in heart rate) and increased myocardial blood flow (Cooke and Tsao 1993). The results of the present study were obtained in the isolated heart and, consequently, the limited neutrophil adhesion could not play a role in the protective effect of the NO donor. Furthermore, we observed no changes in the heart rate or coronary blood flow. Therefore, the reduced myocardial oxygen demand could not contribute to the protective effect observed by us. In our experiments, the protective effect was observed 24 h following a single intravenous injection of NO donor. At this time NO was already undetectable in the heart (EPR-assay). Hence, the protective effect was due to a NO-activated mechanism rather than to NO itself. Taken together these data suggest that activation of HSP70 synthesis can play an important role in the delayed protective effect of NO donors.

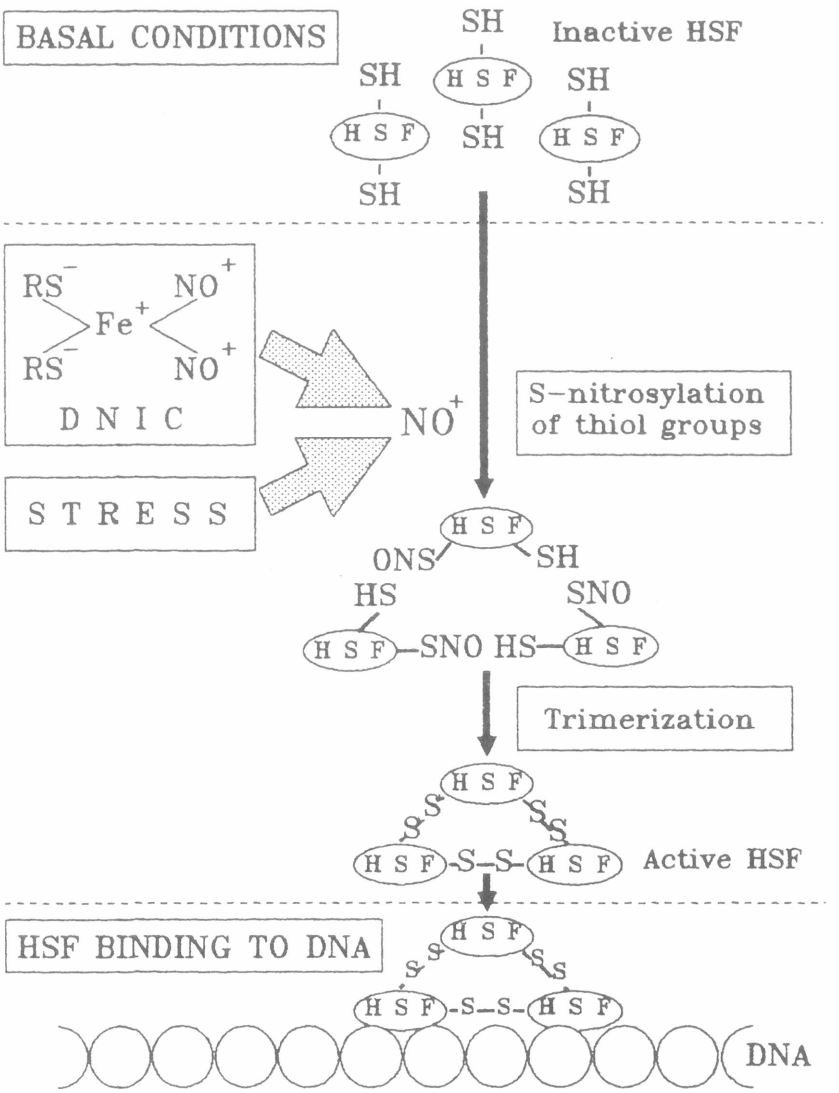

Fig. 4
Tentative interrelations between systems of NO and HSP70 synthesis. For explanation see text.

References

COOKE P.J., TSAO P.S.: Cytoprotective effects of nitric oxide. *Circulation* **88:** 2451–2454,1993.

CURRIE R.M., KARMAZYN M., KLOC M., MAILER K.: Heat shock response is associated with enhanced postischaemic ventricular recovery. *Circ. Res.* **63:** 543–549, 1988.

GILST W.H., KOOMEN J.M.: Calcium repletion-induced arrhythmias after short periods of calcium-free perfusion in the isolated rat heart. *Basic Res. Cardiol.* **82:** 233–240, 1987.

HALL D.M., BUETTNER G.R., MATTHES R.D., GISOLFI C.V.: Hyperthermia stimulates nitric oxide formation: electron paramagnetic resonance detection of NO-heme in blood. *J. Appl. Physiol.* **77:** 548–553, 1994.

HEARSE D.J., HUMPHREY S.M., CHAIN E.B.: Abrupt reoxygenation of the anoxia potassium-arrested perfused rat heart. *J. Mol. Cell. Cardiol.* **5:** 395–401, 1973.

KARMAZYN M., MAILER K., CURRIE R.M.: Acquisition and decay of heat shock enhanced postischaemic ventricular recovery. *J. Physiol. Lond.* **259:** 424–431, 1990.

LAEMMLI V.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.

MEERSON F.Z., MALYSHEV I.Yu.: *Phenomenon of Adaptive Stabilization of Structures and Protection of the Heart* (in Russian) Nauka, Moscow, 1993.

MEERSON F.Z., LAPSHIN A.V., MORDVINCEV P.I., MIKOYAN V.D., MANUKHINA E.B., KUBRINA L.N., VANIN A.F.: Adaptation to stress increases NO generation in animal organs (EPR-investigation) (in Russian), *Bull. Exp. Biol. Med.* **117:** 242–244, 1994.

MONCADA S.: Nitric oxide. *J. Hypertens.* **12** (Suppl. 10): S35–S39, 1994.

MULSCH A., MORDVINTCEV P.I., VANIN A.F.: Quantification of nitric oxide in biological samples by electron spin resonance spectroscopy. *Neuroprotocols* **1:** 165–173, 1992.

PELHAM H.R.B.: Speculation on the functions of the major heat shock and glucose-regulated proteins. *Cell* **46:** 517–528,1986.

SCHLESINGER M.J., ASHBURNER M., TISSIERES A.: *Heat Shock: From Bacteria to Man.* Cold Spring Harbor Laboratory, New York, 1982.

STAMLER J.S.: Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* **78:** 931–936, 1994.

TOMASOVIC S.P.: Functional aspects of the mammalian heat-stress protein response. *Life Chem. Rep.* **1:** 33–63, 1989.

TOWBIN H.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76:** 4350–4354, 1979.

VANIN A.F., MORDVINTCEV P.I., KLESHCHEV A.L.: Appearance of nitric oxide in animal tissue in vivo. *Studia Biophys.* **107:** 135–142, 1984.

VEDERNICOV Y.P., MORDVINTCEV P.I., MALENKOVA I.V., VANIN A.F.: Effect of diethyldithiocarbamate on the activity of nitric oxide-releasing vasodilators. *Eur. J. Pharmacol.* **211:** 313–317, 1992.

WELCH W.J., SUHAN J.P.: Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J. Cell. Biol.* **103:** 2035–2052, 1986.

ZHANG Y.H., TAKAHASHI K., JIANG G.-Z., ZHANG X.-M., KAWAI M., FUKADA M., YOKOCHI T.: In vivo production of heat shock protein in mouse peritoneal macrophages by administration of lipopolysaccharides. *Infect. Immun.* **62:** 4140–4144, 1994.

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