Sodium Azide in Commercially Available C-Reactive Protein Preparations Does Not Influence Matrix Metalloproteinase-2 Synthesis and Release in Cultured Human Aortic Vascular Smooth Muscle Cells

To the Editor:

Detection of circulating concentrations of the acute-phase reactant C-reactive protein (CRP), which is synthesized in response to proinflammatory cytokines, is a relevant tool for identifying the involvement of low-grade inflammation in atherosclerosis and for predicting future atherothrombotic events *(1)*.

Whether CRP is only a marker or is also an active player in atherosclerotic injury is a matter of intense debate *(2)*. CRP is present in atherosclerotic lesions *(3)* and can contribute directly to atherothrombosis *(4)*. In particular, CRP induces expression of proatherogenetic molecules in endothelial cells and promotes LDL uptake by macrophages *(4)*. We recently observed that CRP increases synthesis and secretion of matrix metalloproteinase 2 (MMP-2) from cultured human vascular smooth muscle cells (hVSMCs) *(5)*, a mechanism potentially involved in plaque destabilization.

Recently, however, the reliability of results concerning CRP obtained in vitro has been questioned because commercial CRP preparations contain the biologically active bacteriostatic preservative sodium azide *(6–9)*. In particular, the contaminating presence of sodium azide has been considered responsible for the proapoptotic, antimigratory, antiproliferative, antiangiogenetic, and vasodilating effects previously attributed to CRP *(5–8)*. Thus, it is now necessary to exclude any sodium azide–mediated effect when a new biological action of CRP is described.

Because our study of CRP effects on MMP in VSMCs included the use of a commercial CRP preparation (Sigma Chemical Co.) containing 1 g/L sodium azide, we carried out control experiments to rule out possible effects of sodium azide on MMP-2 synthesis and secretion in VSMCs from microarterioles *(5)*. Our results for aortic hVSMCs were interesting.

Aortic hVSMCs (Cambrex Bioscience Srl) cultured in minimum essential medium supplemented with 100 mL/L fetal calf serum, 100 units/L penicillin, 100 μg/L streptomycin, 10 mmol/L glutamine, and vitamins and buffered with 10 mmol/L TES and 10 mmol/L HEPES were incubated for 24 h with sodium azide at a final concentration of 76.9 μmol/L (concentration reached in culture medium after CRP addition). After incubation, MMP-2 expression (Western blot) and activity (gelatin zymography) were detected with previously described methods *(5)* in both supernatants and cell lysates. Contamination of the cell cultures by lipopolysaccharide was excluded by the *Limulus* assay (Sigma). Endotoxin was also removed from the CRP solution by use of a Detoxigel column (Pierce Biochemicals), as described previously *(10)*. After purification, the endotoxin concentration in the CRP solution was <0.06 endotoxin units (EU)/mL as measured by the *Limulus* assay. The control experiment was performed by incubation of VSMCs with purified CRP solution.

As evidenced in Fig. 1, sodium azide did not influence MMP-2 expression and activity in either cell lysates or supernatants. CRP was used as a positive control. MMP-2 synthesis and secretion were not changed by cell exposure to the lipopolysaccharide antagonist Polymyxin B (0.1 and 0.5 mg/L). Purified CRP increased MMP-2 secretion, expression, and activity, with no difference compared with the nonpurified preparation.

Recent studies underline the need to check the biological effects of a commercial protein preparation derived from a biological source by means of a broad range of control experiments.

The results of our study demonstrate that CRP influences MMP-2 in VSMCs independently of sodium azide, which is present in commercial CRP preparations. To the best of our knowledge, we are the first authors taking into account the effects of sodium azide on MMP-2 synthesis and secretion, providing the first evidence that this agent does not influence MMP-2 synthesis and secretion from hVSMCs.

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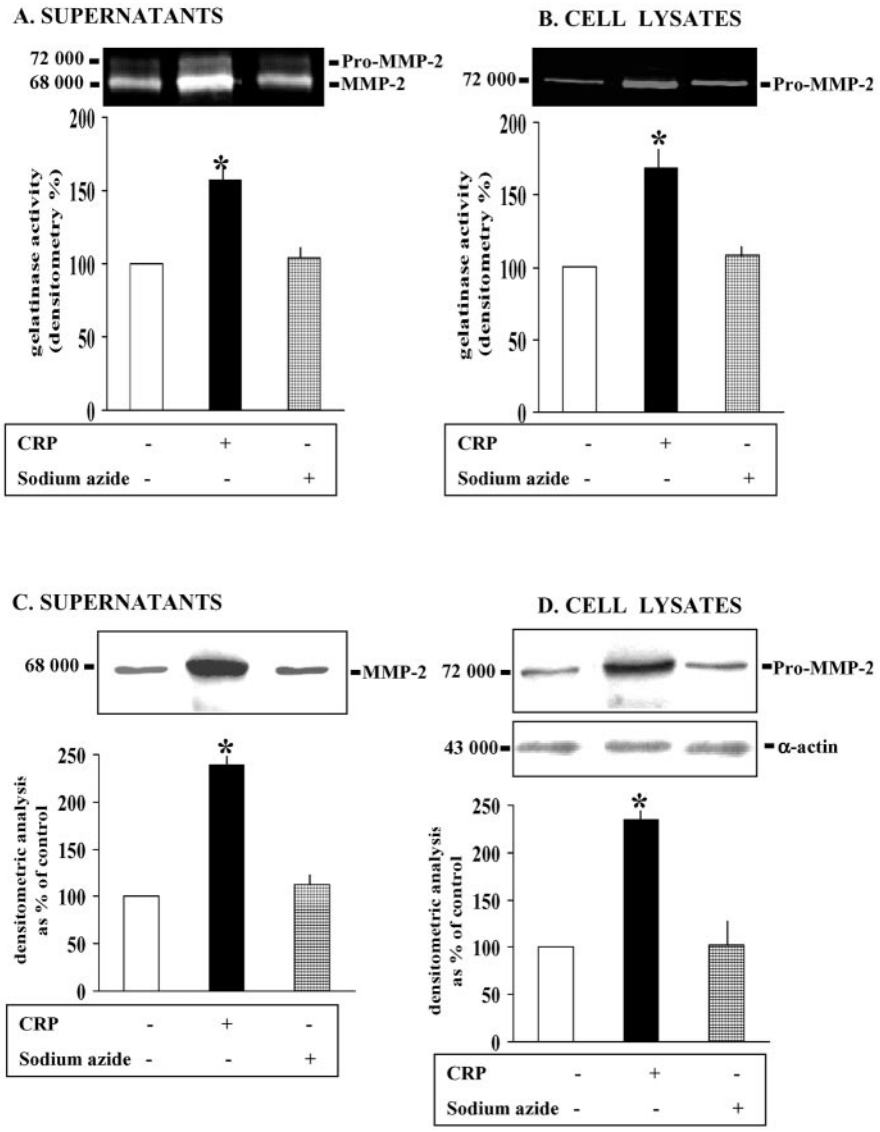

Fig. 1. Effects of sodium azide on expression, secretion, and activity of MMP-2 from hVSMCs.

Densitometric analysis of gelatin zymographs (*A* and *B*) and Western blots (*C* and *D*) of cell supernatants and lysates indicated that CRP (10 mg/L), but not sodium azide (76.9 μmol/L), influences MMP-2 expression, secretion, and activity (*, $P < 0.05$ with CRP and not significant with sodium azide in both supernatants and cell lysates, Student–Newman–Keuls test). Each panel is representative of at least 5 experiments.

Gabriella Doronzo
Isabella Russo
Mariella Trovati
Giovanni Anfossi*

Diabetes Unit
Department of Clinical and Biological Sciences
of the University of Turin
San Luigi Gonzaga Hospital
Orbassano (Turin), Italy

* Address correspondence to this author at: Diabetes Unit, Department of Clinical and Biological Sciences of the Turin University, San Luigi Gonzaga Hospital, I-10043 Orbassano (Turin), Italy. Fax 39-011-9038639; e-mail giovanni.anfossi@unito.it.

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In Vitro Testing for Antiinflammatory Properties of Compounds

To the Editor:

Singh et al. *(1)* recently proposed the application of the human myelomonocytic cell line THP-1 to test for effects of potential antiinflammatory drugs and compounds. THP-1 cells were stimulated with lipopolysaccharide for 4 to 24 h, and the secretion of proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) was assessed. Results showed that various dietary supplements as well as pharmacologic agents significantly inhibited lipopolysaccharide-stimulated TNF-α release *(1)*. Interestingly, this assay matches almost perfectly one we proposed that also uses THP-1 cells but uses neopterin production as a read-out for monitoring potential antiinflammatory effects of compounds *(2)*. After publication of that report, we observed that drugs usually exert more important effects on the T-cell/macrophage interplay than on the stimulated monocytic cells themselves and thereby on the Th1-type cytokine interferon-γ, which is crucially important as a proinflammatory mediator. Unfortunately, because we used only THP-1 cells, potential effects of compounds on the T-cell population were overlooked. Accordingly, we investigated human peripheral blood mononuclear cells (PBMCs) freshly isolated from whole blood of healthy donors and stimulated them with mitogens *(3–6)*. PBMCs were seeded at a density of 1.5×10^6 cells/mL and preincubated with compounds for 30 min before stimulation with phytohemagglutinin or concanavalin A. We found that a mitogen concentration of 10 μg/mL was optimal for detecting suppressive effects of compounds. Cells were incubated for 48 h at 37 °C and 5% CO_2, and supernatants were collected thereafter. Measurements of neopterin formation by methods such as ELISA and/or tryptophan degradation by HPLC were used as convenient readouts; both biochemical effects are in-