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INDUCTION OF STRESS PROTEINS IN MOUSE PERITONEAL MACROPHAGES BY THE ANTI-RHEUMATIC AGENTS GOLD SODIUM THIOMALATE AND AURANOFIN

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Abstract—Gold sodium thiomalate and auranofin, anti-rheumatic gold-containing compounds, induced some stress proteins in cultured mouse peritoneal macrophages. The enhanced synthesis of two proteins, heme oxygenase (a 34-kDa protein) and a 23-kDa protein, was particularly prominent. The 23-kDa protein induced by the gold compounds was identical to that found in macrophages exposed to oxidative stress and was suggested to have antioxidant activity. Intraperitoneal injection of gold sodium thiomalate and oral administration of auranofin to mice induced enhanced synthesis of these proteins in peritoneal macrophages analyzed *ex vivo*. These data suggest that increased synthesis of these proteins may have a role in mediating the pharmacologic effect of these agents.

Key words: gold sodium thiomalate; auranofin; rheumatoid arthritis; stress proteins; heme oxygenase; macrophage

Mammalian cells respond to a wide variety of adverse conditions by inducing the synthesis of a number of stress proteins. We have reported the induction of 34- and 23-kDa stress proteins in mouse peritoneal macrophages by oxidative and sulfhydryl-reactive agents [1]. The 34-kDa protein has been identified as heme oxygenase [2], whereas the function of the 23-kDa protein is unknown. However, cDNA encoding the 23-kDa protein has been cloned, and sequence analysis of the cDNA revealed that it has high homology to the C22 component of alkyl hydroperoxide reductase of *Salmonella typhimurium* [3]. This enzyme is induced by H_2O_2 under the control of a regulatory gene, *oxy R* [4], and its function would be the detoxification of lipid and other hydroperoxides that are produced during oxidative stress [5]. Thus, it seems that the 23-kDa stress-induced macrophage protein, designated MSP23†, is involved in a defense system against oxidative stress. Gold sodium thiomalate and auranofin are used frequently in the treatment of rheumatoid arthritis, and investigators have described both humoral and cellular components as the targets of these compounds [6–11]. Macrophages are assumed to be a target of these drugs because the drugs have been shown to have several inhibitory effects on macrophage function [8]. The challenge of several mammalian cell types in culture with auranofin results in increased synthesis of 32- or 34-kDa proteins presumed to be heme oxygenase [12, 13]. In the present study, we report the induction of stress proteins by gold sodium thiomalate and auranofin in mouse peritoneal macrophages analyzed *in vivo* and *ex vivo* after drug treatment.

MATERIALS AND METHODS

Auranofin was a gift from Smithkline Beecham Japan. Gold sodium thiomalate was obtained from the Shionogi Pharmaceutical Co., Ltd. Auranofin was dissolved in ethanol at 2 mM and dispensed to the cultured macrophages. For oral administration, auranofin was suspended in water at 4 mg/mL. A stock solution of gold sodium thiomalate was made by dissolving it in saline at 25 mM (approximately 10 mg/mL) and was used throughout.

Macrophages were collected by peritoneal lavage from female C57BL/6N mice, weighing 20–25 g, that had received an i.p. injection of 2 mL of 4% thioglycollate broth 4 days previously. The cells were washed twice with RPMI-1640 medium, plated at 1×10^6 cells per 35-mm diameter culture dish in RPMI-1640 medium containing 10% fetal bovine serum, and were incubated at 37° in 5% CO_2 in air. After 1 hr the medium was changed to a fresh one to remove non-adherent cells. The cells in the dish were treated with or without gold compounds in RPMI-1640 medium containing 10% serum, and then the cell proteins were radiolabeled by incubating

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† Abbreviation: MSP23, the 23-kDa macrophage stress protein.

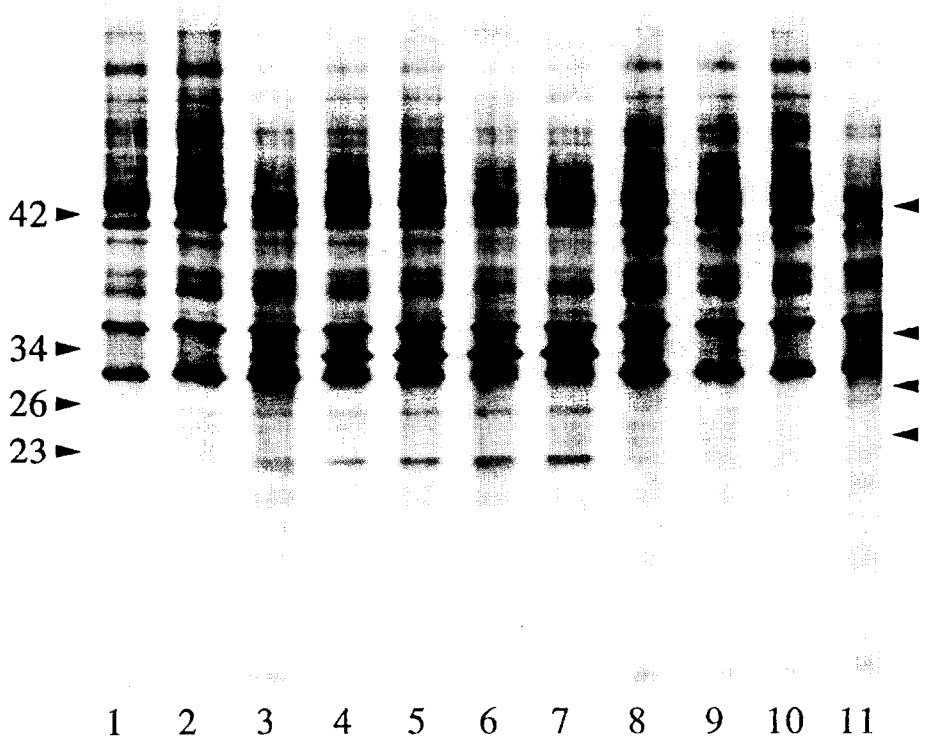

Fig. 1. Induction of stress proteins in macrophages by gold sodium thiomalate. Lanes 1–7: peritoneal macrophages were incubated for 11 hr with gold sodium thiomalate at 0 (lane 1), 2.5 μM (lane 2), 12.5 μM (lane 3), 50 μM (lane 4), 125 μM (lane 5), 250 μM (lane 6), and 500 μM (lane 7). Lanes 8–11: the macrophages were incubated for 11 hr with sodium thiomalate at 10 μM (lane 8), 50 μM (lane 9), 200 μM (lane 10), and 500 μM (lane 11). The arrows indicate 23-, 26-, 34-, and 42-kDa proteins.

macrophages for 30 min in 1 mL of methionine- and cysteine-free RPMI-1640 medium containing 10% dialyzed fetal bovine serum and 50 μCi/mL ^{35}S-protein labeling mixture (EXPRE^{35}S^{35}S Protein Mix from DuPont-New England Nuclear, sp. act. > 1000 Ci/mmol, containing > 77% L-[^{35}S]methionine and 18% L-[^{35}S]cysteine). The radioactive medium was then discarded, and the cells were washed with phosphate-buffered saline, lysed, and analyzed by SDS–PAGE and fluorography as described previously [1]. Immunoblot analysis was performed as described previously [2], using an antibody raised against rat heme oxygenase or against MSP23.

RESULTS

Exposure of macrophages isolated from the peritoneal cavity with gold sodium thiomalate enhanced the synthesis of several proteins, e.g. 34-, 26-, and 23-kDa proteins (Fig. 1). Among them the 34- and 23-kDa proteins were prominently induced. The minimum concentration of gold sodium thiomalate required to induce detectable synthesis of stress proteins was 12.5 μM. Gold was essential to this induction because thiomalate failed to enhance production of the stress proteins. It should be noted that the synthesis of the 42-kDa protein, presumably actin, was suppressed by gold sodium thiomalate in a concentration-dependent manner. Figure 2 shows a fluorograph of the time course of synthesis of the stress proteins after exposure to gold sodium thiomalate. The elevated synthesis of the 34-kDa protein preceded that of the 23-kDa protein. After a 24-hr exposure, the synthesis of the 34-kDa protein was almost undetectable and, concurrently, the suppressed synthesis of the 42-kDa protein was recovered. Induction of stress protein synthesis in cultured macrophages by auranofin is shown in Fig. 3. This triethylphosphine gold compound was much more potent in the induction than gold sodium thiomalate. Detectable synthesis of 34- and 23-kDa proteins was induced by auranofin at 0.1 μM. The time course of the induction of the stress proteins by auranofin (data not shown) was almost the same as that by gold sodium thiomalate shown in Fig. 2. In addition, these time courses of induction by the gold compounds were quite similar to that by diethyl maleate previously reported [1], suggesting that the uptake of these compounds by macrophages is not rate-limiting in the induction of stress protein synthesis. It should be noted that macrophages were mostly viable (> 95%), as judged by the trypan blue exclusion method, during the exposure of the gold compounds in the above experiments. However, at concentrations above 500 μM gold sodium thiomalate or 2 μM auranofin, they were considerably cytotoxic, and viability decreased within 12 hr.

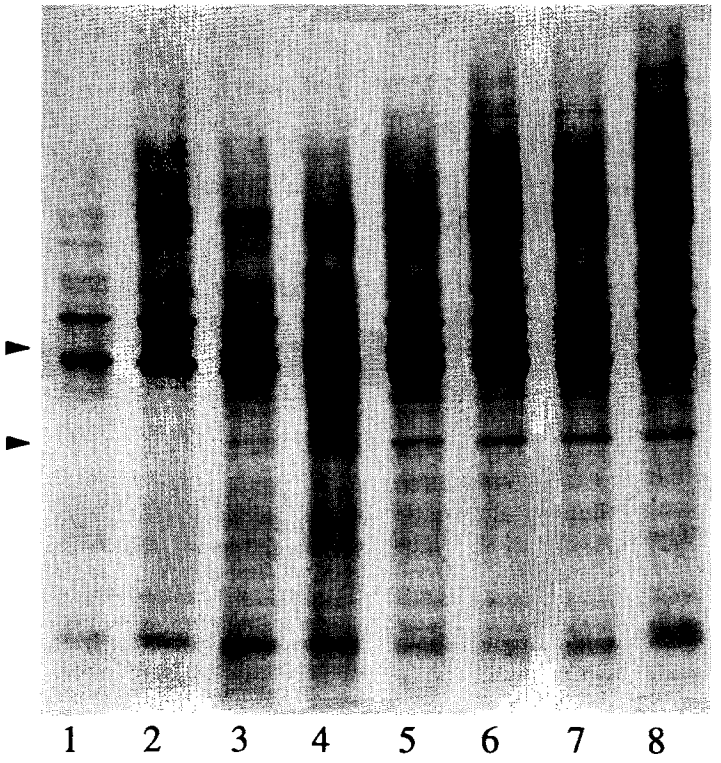

Fig. 2. Time course of induction of stress proteins in macrophages by gold sodium thiomalate. Peritoneal macrophages were incubated with 50 μM gold sodium thiomalate for 0 hr (lane 1), 2 hr (lane 2), 5 hr (lane 3), 8 hr (lane 4), 11 hr (lane 5), 14 hr (lane 6), 17 hr (lane 7), and 23 hr (lane 8). The arrows indicate 23- and 34-kDa proteins.

To identify the 34- and 23-kDa proteins, immunoblot analysis was performed. As shown in Fig. 4, the 34-kDa protein reacting with the anti-heme oxygenase antibody increased greatly after exposure of the cells to gold sodium thiomalate or auranofin. We prepared antibody raised against MSP23, which was induced in macrophages by oxidative or sulfhydryl reactive agents [1]. Immunoblot analysis demonstrated that the protein reacting with this antibody increased by challenge of the cells with gold sodium thiomalate or auranofin. The increase in the amount of heme oxygenase or MSP23 provoked by the gold compounds was estimated by densitometric analysis of the immunoblot pattern shown in Fig. 4. In comparison with the control, i.e. in the absence of gold compounds (lane 2), heme oxygenase was increased by 17- and 25-fold when the cells were exposed to gold sodium thiomalate and auranofin, respectively. Similarly, MSP23 was increased by 2.5- and 3-fold when the cells were exposed to gold sodium thiomalate and auranofin, respectively.

Intraperitoneal injection of gold sodium thiomalate or oral administration of auranofin to mice induced the synthesis of several proteins in peritoneal macrophages analyzed *ex vivo* (Fig. 5). The synthesis of the 34- and 23-kDa proteins was strongly enhanced by *in vivo* injection of gold sodium thiomalate, whereas the induction of the synthesis was rather weak in macrophages prepared from mice orally administered auranofin.

DISCUSSION

It is generally accepted that rheumatoid arthritis is associated with deficiencies in the regulation of the immune response that result in excessive inflammation and irreversible deterioration of joints. Gold [Au(I)] compounds are slow-acting anti-rheumatic drugs, and a characteristic feature of therapy with these drugs is the delayed onset of a clinical effect. Although the molecular basis for such therapeutic action is unclear, these compounds appear to be active by virtue of their capacity to depress various functions of mononuclear phagocytes. *In vitro* studies have demonstrated that they inhibit antigen-induced proliferation of lymphocytes indirectly by inhibiting the accessory function of monocytes [7]. It has also been shown that they inhibit the differentiation of monocytes into effector cells [9].

The present experiments demonstrated that the gold compounds induced the synthesis of heme oxygenase and MSP23 in macrophages, resulting in the accumulation of these proteins. Heme oxygenase plays an essential role in heme catabolism by cleaving heme to form biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. An increase in heme oxygenase activity will increase cellular capacity to generate both biliverdin and bilirubin providing that biliverdin reductase is present. Since bilirubin can react with O_2^- and many of the potential cellular forms of the products of

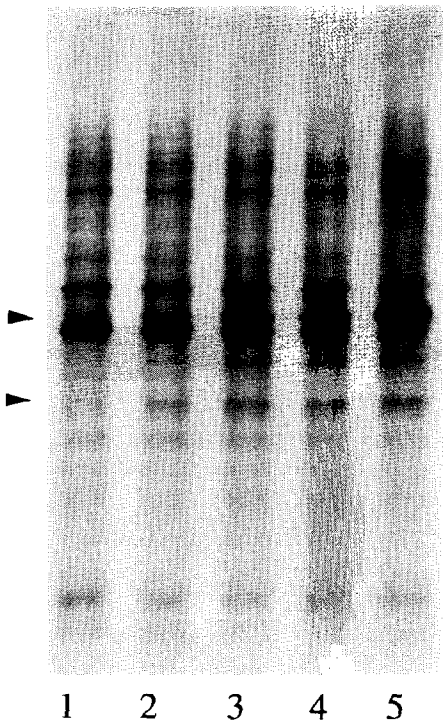

Fig. 3. Induction of stress proteins in macrophages by auranofin. Peritoneal macrophages were incubated for 11 hr with auranofin at 0 (lane 1), 0.1 μM (lane 2), 0.5 μM (lane 3), 1 μM (lane 4), and 2 μM (lane 5). Auranofin was dissolved in ethanol, and the final concentration of ethanol in all samples was 0.1%. The arrows indicate 23- and 34-kDa proteins.

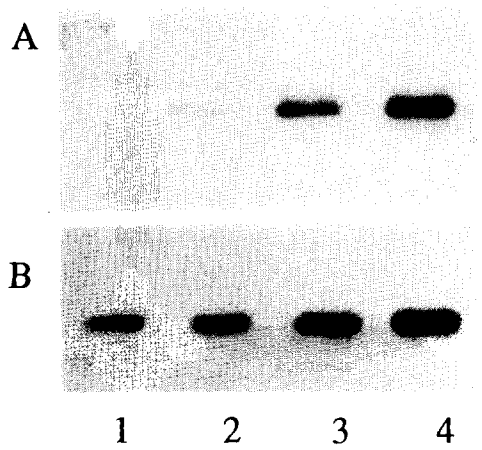

Fig. 4. Induction of heme oxygenase and MSP23 in macrophages by gold sodium thiomalate and auranofin. Peritoneal macrophages were not incubated (control, lane 1), or incubated for 11 hr without any gold compounds (lane 2), with 50 μM gold sodium thiomalate (lane 3), or with 0.5 μM auranofin (lane 4). The proteins were electrophoresed and analyzed by immunoblotting. (A) immunostaining by anti-heme oxygenase antibody; (B) immunostaining by anti-MSP23 antibody.

heme catabolism react efficiently with peroxyl radicals [14], these products may play a direct role in cellular defense against oxidative damage. The cells may develop tolerance to the oxidant by the induction of heme oxygenase.

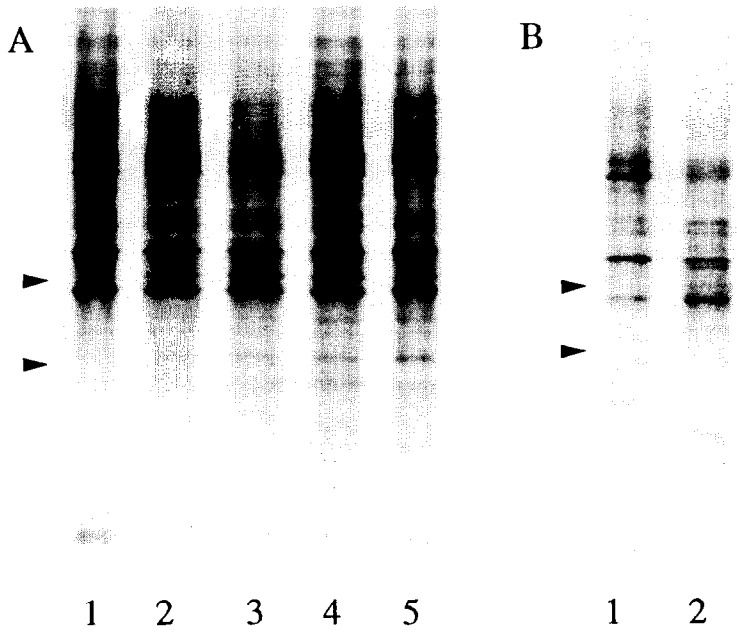

Fig. 5. *Ex vivo* analysis of stress proteins in macrophages. Mice previously treated with thioglycollate broth as described in Materials and Methods were injected i.p. with gold sodium thiomalate (panel A) at 0 (lane 1), 0.1 mg/mouse (lane 2), 0.25 mg/mouse (lane 3), 0.5 mg/mouse (lane 4), and 1 mg/mouse (lane 5), or administered auranofin orally (panel B) at 0 (lane 1), and 2 mg/mouse (lane 2). Peritoneal cells were harvested at 10 hr following administration of drugs, plated, and cultured for 1 hr to remove non-adherent cells, and then were radiolabeled.

The biological function of MSP23 is largely unknown. However, proteins sharing homology with MSP23 have recently been found in bacteria from eukaryotes [15]. For example, an antioxidant protein sharing 73% homology with MSP23 has been found in yeast [16]. This protein prevents the inactivation of various enzymes caused by a nonenzymatic Fe^{3+}/O_2/thiol mixed-function oxidation system but not by mixed-function oxidation systems in which the thiol component is replaced by another electron donor [17]. When the thiol component acts as an electron donor, reactive sulfur species, such as thiyl radicals, are produced, in addition to the reactive oxygen species. Since the protective activity of this protein is specific to thiol-containing systems, this protein is named "thiol-specific antioxidant" which may be capable of eliminating reactive sulfur species [16]. In addition to this yeast protein, a cDNA, corresponding to a gene that shares 95% identity with MSP23 at the amino acid level and therefore, perhaps, a human homologue of MSP23, has been cloned from a *ras*-transformed human mammary epithelial cell line [18]. A protein presumably identical to that encoded by this cDNA has been purified from red blood cells. This protein also has antioxidant activity and blocks inactivation of enzymes by the thiol-containing oxidation system [19]. Thus, the induction of MSP23 may afford enhanced protection against oxidative stress to the cells. It should be noted that the 34- or 32-kDa protein, presumably heme oxygenase, has been induced in various types of cells, whereas the induction of the 23-kDa protein has not been observed in cells except for monocytes [12].

Since heme oxygenase and MSP23 are stress proteins in macrophages, gold compounds are considered as stress agents. When they are administered *in vivo*, they accumulate in the body and may serve as stress agents. Induction of the stress proteins in macrophages may be involved in the reduced activation and the inhibited maturation of macrophages provoked by the gold compounds.

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