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Research Article

Geranylgeranylacetone protects human monocytes from mitochondrial membrane depolarization independently of Hsp70 expression

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Abstract. The anti-ulcer drug geranylgeranylacetone (GGA) has been shown to induce the expression of heat shock proteins (HSPs), in particular of Hsp70, in gastric and small intestine cells. In this study, we investigated whether GGA was able to induce Hsp70 in another cell type, human monocytes, which represent a well-established model of Hsp70 expression under oxidative stress. In these cells, GGA had no significant effect either on basal or tobacco smoke-induced Hsp70 expression. We further investigated the effects of GGA on mitochondria, a key organelle of oxidant-mediated cell injury and a putative target for GGA-mediated protection. GGA significantly increased basal mitochondrial membrane polarization and inhibited the decrease in mitochondrial membrane potential of human monocytes exposed to distinct sources of clinically relevent oxidants such as tobacco smoke and γ-irradiation. Our results indicate that mitochondria are targets for GGA-mediated protection against oxidative stress in human monocytes, independently of Hsp70.

Key words. Oxidative stress; protection; Hsp70; mitochondria; geranylgeranylacetone.

Geranylgeranylacetone (GGA) (Selbex, Eisai Co., Japan), an acyclic polyisoprenoid, has been used as an anti-ulcer drug since 1981 [1, 2]. GGA protects the gastric mucosa and small intestine against lesions induced by a large variety of insults, including ethanol, aspirin, cold stress, and indomethacin [2–7]. Although the protective effects of GGA are well established, the mechanism(s) underlying this protection are not yet fully elucidated. GGA stimulates mucus secretion and phospholipid production [2, 5, 8] and induces the synthesis of heat shock (HS)/stress protein (HSP) and, in particular, the cytosolic, inducible, highly protective, 72-kDa HSP, Hsp70 [9–11].

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HSPs are induced as an adaptative response in all cells and organisms upon exposure to a number of stresses, in particular, oxidative stress. Oxidative stress may be caused by environmental exposures such as tobacco smoke (TS) or γ-irradiation, and is involved in the emergence of diseases and conditions such as cancer, cardiovascular diseases, and aging. HSPs function as molecular chaperones involved in the correct folding and refolding of proteins, and thereby exert essential housekeeping and protective functions. HSPs contribute to cell resistance and survival under stressful conditions such as exposure to HS, heavy metals, ethanol, and reactive oxygen species (ROS) [12–17]. The induction of Hsp70 by GGA directly correlates with protection against ethanol-induced damage in cultured guinea pig gastric mucosa [9] and with a reduction in intestinal ischemic injury in rats [10]. Thus, GGA-mediated Hsp70 induction could relate to the pro-

tective effects of the drug under stress conditions. Here we investigated whether GGA-mediated induction of Hsp70 extended to human peripheral blood mononuclear cell-isolated from the blood of healthy donors. These cells display a high sensitivity to oxidants and represent a well-established model to analyze TS-mediated damage and subsequent Hsp70 expression [18–20]. As the lipid peroxidation-inhibitor α-tocopherol (vitamin E) is systematically added to GGA preparations to prevent the oxidation of GGA, we always compared, in our studies, the effects of GGA to those of α-tocopherol alone. In human monocytes, however, neither GGA nor α-tocopherol increased Hsp70 expression, under basal conditions or after TS exposure.

We thus hypothesized that GGA could exert its effects via mechanisms distinct from Hsp70 induction. Mitochondria play a key role in cellular responses to oxidative stress and in oxidant-mediated cell death [21], notably upon exposure to TS [20, 22]. Moreover, they have been suggested to be central to the aging process [23, 24]. Mitochondrial membrane potential ($\Delta\psi$m) represents an adequate measure of mitochondrial function [25]. We thus investigated the effects of GGA on $\Delta\psi$m under normal and stressful conditions. Human monocytes were exposed to TS or γ-irradiation as relevant environmental and clinical sources of oxidants [26]. GGA, but not α-tocopherol, increased basal $\Delta\psi$m and prevented $\Delta\psi$m disruption upon exposure to TS or γ-irradiation. Our results highlight mitochondria as a novel target for the protective effects of GGA.

Materials and methods

Cells

Human peripheral blood mononuclear cells from healthy donors (buffy coats) were isolated by gradient centrifugation and purified by adherence. Cells (2.5×10^6 cells/ml) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, in a humidified atmosphere containing 5% CO_2 in air, at 37 °C.

Treatments

1) GGA treatment. GGA (Selbex, EisaiI Co., Tokyo, Japan) is highly sensitive to oxidants and thus GGA preparations always contain α-tocopherol (0.02%). Cells were exposed to α-tocopherol alone [0.02%, dissolved in dimethyl sulfoxide, (DMSO)] or GGA (1 µM), also containing 0.02% α-tocopherol. DMSO was used as vehicle in the control samples. To study the effect of α-tocopherol or GGA on TS-mediated Hsp70 expression or mitochondrial membrane potential depolarization, monocytes were pre-incubated with α-tocopherol alone (0.02%) or GGA (1 µM) (containing α-tocopherol) for 2 h before TS treatment or cell irradiation.

2) TS exposure. A peristaltic pump-smoke machine (Heinr. Borg-waldt RM1/G, Hamburg, Germany) was used to generate TS-bubbled phosphate-buffered saline (PBS) from mainstream smoke of standard research cigarettes (reference 2R1, University of Kentucky) through a puffing mechanism related to the human smoking pattern: one puff/min (one puff = 35 ml), each puff of 2 s duration. In the experiments presented here, the aqueous smoke fractions of one cigarette corresponded to 10 puffs (350 ml) bubbled into 5 ml of PBS. The final dilutions were expressed as puff/ml of culture medium. After performing extensive dose-response experiments [18], we used concentrations ranging from 0.06 to 0.12 puff/ml. Monocytes were exposed to TS-bubbled PBS for 4 h, then washed twice in PBS, and recovered by gentle scraping for Hsp70 and $\Delta\psi$m detection.

3) Irradiation. Irradiation was performed at room temperature, in air, using a γ-ray source (^{137}Cs, irradiator IBL637) at a fixed dose rate of 2 Gy/min. All cells were exposed to 20 Gy and allowed to recover for 24 or 48 h before being harvested by scraping. Cells were then collected and $\Delta\psi$m was analyzed by flow cytometry.

4) HS treatment. HS (30 min at 44 °C, followed by 4 h of recovery at 37 °C) was used as a positive control for Hsp70 expression.

Detection of Hsp70 expression

Hsp70 expression was detected by flow cytometry as previously described [19]. Briefly, both permeabilized and nonpermeabilized monocytes were incubated with the human anti-Hsp70 antibody (SPA 810; Stressgen, Victoria, Canada) and stained with rabbit anti-mouse /FITC as previously described [19]. Analysis was performed using a EPICS XL flow cytometer (Coulter, Miami, Fla.). A total of 5000 cells per sample were acquired in listmode and analyzed with the Elite software version 4,02.

$\Delta\psi$m detection

$\Delta\psi$m detection was performed using the fluorescent probe JC-1 (Molecular Probes, Eugene, Ore.) as described elsewhere [27, 28]. The lipophilic cation JC-1 forms aggregates in the matrix of intact mitochondria (emitting at 590 nm). Thus, mitochondrial membrane depolarization is associated with a shift in JC-1 fluorescence emission, from red to green. Cells were incubated for 10 min at 37 °C in the dark with 0.5 ml PBS containing JC-1, 50 ng/ml. After washing, cells were analyzed by flow cytometry. Five thousand cells per sample were counted in acquisition mode and analyzed using Elite 4,01 software.

Statistical analysis

Statistical comparisons were made by Student’s t-test.

Results

Effects of GGA on Hsp70 expression in human monocytes

As GGA has been previously shown to induce Hsp70 in gastric and small intestine cells, we tested whether it also increased Hsp70 expression in normal human monocytes isolated from the blood of healthy donors and cultured in the presence of 10% fetal calf serum. A positive control for Hsp70 expression was performed by heat shocking the cells for 30 min at 44 °C. Because GGA solution contains α-tocopherol, we also tested the effects of α-tocopherol alone. Cells were incubated with α-tocopherol (0.02%) or GGA (1 μM GGA and 0.02% α-tocopherol) for 2–6 h. At each 45 min interval, Hsp70 expression was determined. Although GGA increased Hsp70 within some individual experiments (not shown), overall the drug had no significant effect on Hsp70 expression (fig. 1A); neither did α-tocopherol.

We also investigated whether GGA modulated Hsp70 induction after oxidative stress. Our knowledge about human monocyte responses to the oxidant-mediated toxicity of TS motivated our study on the effects of GGA on TS-mediated Hsp70 expression [18, 20, 22]. Cells were incubated with α-tocopherol or GGA solution (1 μM GGA and 0.02% α-tocopherol) for 2 h before being exposed to TS (0.06 and 0.12 puff/ml) for 4 h. TS significantly increased Hsp70 expression in human monocytes, while the GGA preparation and α-tocopherol had no effect on TS-mediated Hsp70 inducibility for either TS concentration (fig. 1B).

Effects of GGA on basal $\Delta\psi$m disruption

To test whether GGA could protect cells by preserving mitochondrial functions, we investigated, in the same cells, the effects of GGA on basal $\Delta\psi$m. Human monocytes from healthy donors were incubated with GGA (1 μM) or α-tocopherol (0.02%) for 2 h. The percentage of cells with depolarized mitochondria was then determined by flow cytometry. According to extended studies performed in more than 100 donors, basal $\Delta\psi$m varies among individuals, with 5–30% of monocytes presenting depolarized mitochondria under basal conditions (M. Vayssier-Taussat, Y. Aron and B. S. Polla, unpublished data). The data presented in figure 2 show the results obtained with cells from three donors, for whom the percentage of cells with depolarized mitochondria ranged from 19 to 27%. For those and all other tested donors (n = 8), GGA pre-treatment lowered the percentage of cells with depolarized mitochondria. This effect was due to GGA alone, since α-tocopherol had no effect on mitochondrial membrane potential in all donors. Moreover, addition of GGA just before the analysis did not affect the percentage of cells with depolarized mitochondria (data not shown).

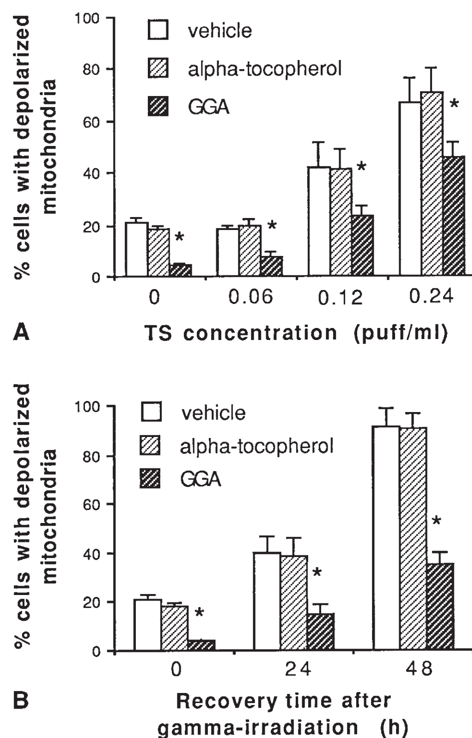

Figure 1. Effects of GGA on Hsp70 expression of human monocytes. (*A*) Human peripheral blood mononuclear cells from healthy donors were exposed to α-tocopherol alone (0.02%, dissolved in DMSO) or GGA (1 μM), also containing 0.02% α-tocopherol, for the incubation times indicated. DMSO was used as vehicle in the control samples and HS (30 min at 44 °C, followed by 4 h of recovery) was used as a positive control for Hsp70 expression. Hsp70 expression was detected by flow cytometry. Values are means ± SD of percent cells expressing Hsp70 (n = 6; *p < 0.01, HS-treated cells compared to control). (*B*) Monocytes were pre-incubated with α-tocopherol alone (0.02%) or GGA (1 μM) (containing α-tocopherol) for 2 h before being exposed to TS-bubbled PBS for 4 h. Cells were then collected and labelled with anti-human Hsp70. Hsp70 expression was analyzed by flow cytometry. Values are expressed as percentage cells expressing Hsp70 ± SD (n = 4; *p < 0.01, TS-treated cells compared to control).

Effects of GGA on oxidant-induced $\Delta\psi$m disruption

In view of the selective effects of GGA on basal $\Delta\psi$m, we also investigated the effects of GGA on oxidant-mediated $\Delta\psi$m disruption. After 2 h of pre-incubation with α-tocopherol or GGA, monocytes were exposed to TS or γ-irradiation. Cells were exposed to TS at concentrations ranging from 0.06 to 0.24 puff/ml for 4 h before $\Delta\psi$m analysis (fig. 3A). As already described, TS induced a dose-dependent decrease in $\Delta\psi$m, i.e., an increase in the percentage of cells with depolarized mitochondria [22]. This effect was significantly reduced by GGA pre-treatment for TS concentrations of 0.06 and 0.12 puff/ml (n = 5, p < 0.01). The protection was clearly due to GGA alone, since α-tocopherol did not affect TS-mediated $\Delta\psi$m disruption. We then tested the effect of GGA on $\Delta\psi$m of irradiated cells by pre-treating human monocytes with α-tocopherol alone (0.02%) or with GGA (1 μM)

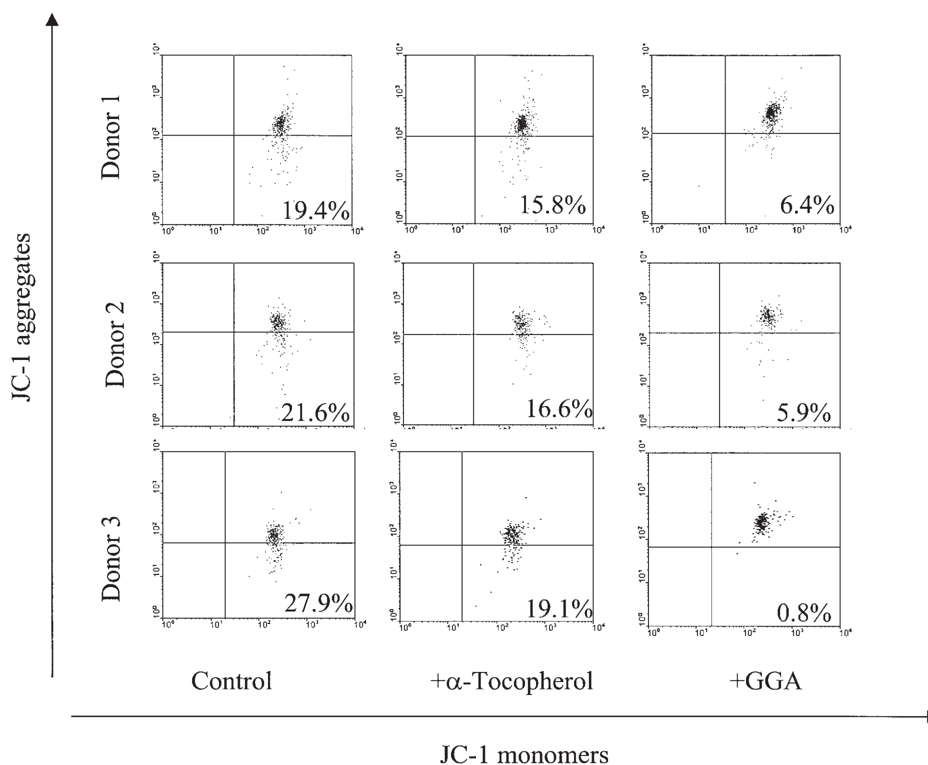

Figure 2. Effects of GGA on basal $\Delta\psi$m in human monocytes. Monocytes were incubated with α-tocopherol alone (0.02%) or GGA and α-tocopherol (1 µM GGA and 0.02% α-tocopherol) for 2 h. Cells were collected before $\Delta\psi$m analysis by flow cytometry (n = 3).

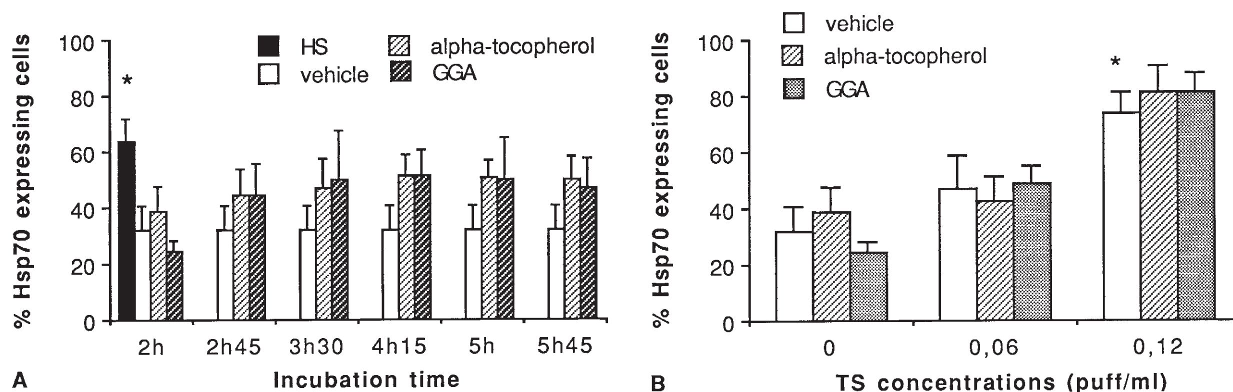

Figure 3. Effects of GGA on oxidant-mediated $\Delta\psi$m disruption in human monocytes. (*A*) Effects of GGA on TS-mediated $\Delta\psi$m disruption in human monocytes. Monocytes were incubated with α-tocopherol alone (0.02%) or GGA with α-tocopherol (1 µM and 0.02%, respectively) for 2 h. Cells were then exposed to TS (0.06, 0.12, and 0.24 puff/ml) for 4 h, then collected and analyzed by flow cytometry. Values are means ± SD of percent cells with depolarized mitochondria. Statistical comparisons were made by comparing TS-treated cells with TS-treated cells pre-treated with α-tocopherol and GGA (n = 5; *p<0.01, GGA-treated cells compared to vehicle or α-tocopherol for each TS concentration). (*B*) Effects of GGA on γ-irradiation-mediated $\Delta\psi$m disruption in human monocytes. Monocytes were incubated with α-tocopherol alone (0.02%) or with GGA and α-tocopherol (1 µM and 0.02%, respectively) for 2 h before irradiation. After indicated recovery times $\Delta\psi$m was analyzed by flow cytometry. Values are means ± SD of percent cells with depolarized mitochondria. Statistical comparisons were made by comparing irradiated-treated cells with irradiated-treated cells pre-treated with α-tocopherol and GGA (n = 5 for controls and n = 4 for irradiated cells; *p<0.001, GGA treated cells compared to vehicle or α-tocopherol at each time point after irradiation).

for 2 h before irradiation (20 Gy). $\Delta\psi$m disruption was analyzed 24 and 48 h after irradiation (fig. 3B). As described for TS exposure, GGA, but not α-tocopherol, prevented γ-irradiation-mediated $\Delta\psi$m disruption at both 24 and 48 h post-irradiation (n = 4, $p<0.01$).

Discussion

The present study provides the first evidence indicating that GGA may exert its protective effects on mitochondria, a mechanism of protection independent of HSP induction.

According to previous studies, the protective effect of GGA on gastric and intestinal cells is due, at least in part, to the induction of Hsp70. However, in human monocytes isolated from healthy donors, GGA did not significantly induce Hsp70, indicating that HSP induction by GGA cannot be extended to all cell models and probably depends on the cell type and the cellular environment. Indeed, all our experiments were performed in the presence of 10% fetal calf serum, while Hirakawa et al. [9] serum-starved cells overnight before GGA treatment and detection of Hsp70. The effects of GGA on Hsp70 induction in a serum-free environment is probably distinct from its effects in the presence of serum. However, the fact that GGA is also an inducer of Hsp70 in vivo suggests that environmental factors distinct from serum, such as cell type, are important for GGA-mediated Hsp70 induction [10, 11].

GGA selectively increased basal mitochondrial membrane potential ($\Delta\psi$m) and protected human monocytes against TS- and γ-irradiation-mediated $\Delta\psi$m disruption. When GGA was added just before the analysis, it did not affect the percentage of cells with depolarized mitochondria, removing the possibility that GGA could react with the JC-1 probe instead of affecting mitochondrial depolarization.

We have previously shown that in the human pre-monocytic line U937, Hsp70 can protect against oxidative stress by protecting mitochondria [29]. As, however, GGA had no detectable effect on Hsp70 induction in human monocytes, GGA-mediated protection of mitochondria is unlikely to relate to Hsp70. Moreover, GGA preparations contain the antioxidant α-tocopherol, a well-established inhibitor of lipid peroxidation. α-Tocopherol had no effect on $\Delta\psi$m in either control cells or cells exposed to oxidants. These results suggest that the effects of GGA on mitochondrial protection are also independent of a potential anti-oxidant effect of the drug. This hypothesis is further supported by the fact that GGA also increased $\Delta\psi$m in control human monocytes which were not exposed to oxidative stress in vitro. We thus propose that GGA acts directly on mitochondria themselves. It will be of interest to further establish, using electrochemical approaches to quantify mitochondrial respiration, whether a specific respiratory complex, or mitochondrial enzyme, is the relevant target for GGA-mediated protection.

Finally, as a ROS-mediated decline in mitochondrial functions has been linked to the aging process [23, 24], protecting mitochondria could theoretically influence aging. If our in vitro findings on the protective effects of GGA on mitochondria both under basal conditions and upon exposure to ROS are confirmed by in vivo studies, GGA could be classified as a novel, mitochondrially targeted, pharmacologically active, protective agent.

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1 Murakami M., Oketani K., Fujisaki H., Wakabayashi T. and Ohgo T. (1981) Antiulcer effect of geranylgeranylacetone, a new acyclic polyisoprenoid on experimentally induced gastric and duodenal ulcers in rats. Arzneimittelforschung **31:** 799–804

2 Terano A., Hiraishi H., Ota S. and Sugimoto T. (1986) Geranylgeranylacetone, a novel anti-ulcer drug, stimulates mucus synthesis and secretion in rat gastric cultured cells. Digestion **33:** 206–210

3 Murakami M., Oketani K., Fujisaki H., Wakabayashi T., Inai Y., Abe S. et al. (1983) Effect of synthetic acyclic polyisoprenoids on the cold-restraint stress induced gastric ulcer in rats. Jpn. J. Pharmacol. **33:** 549–556

4 Oketani K., Murakami M., Fujisaki H. and Wakabayashi T. (1983) Effect of geranylgeranylacetone on aspirin-induced changes in gastric glycoproteins. Jpn. J. Pharmacol. **33:** 593–601

5 Bilski J., Murty V. L. N., Nadziejko C., Sarosiek J., Aono M., Moriga M. et al. (1988) Protection against alcohol-induced gastric mucosal injury by geranylgeranylacetone: effect of indomethacin. Digestion **41:** 22–33

6 Hachiya A., Bessho M., Iwasaki T., Iida K. and Otsuka S. (1996) Protective effect of teprenone on blood flow and incidence of histologic lesions in rat gastric mucosa after hemorrhage and retransfusion. Scand. J. Gastroenterol. **31:** 326–333

7 Terano A., Shiga J., Mutoh H., Hiraishi H., Shiina S., Kurita M. et al. (1991) Tetraprenylacetone promotes healing process of ethanol-induced gastric damage in the rat. Jpn. J. Pharmacol. **55:** 115–120

8 Nishizawa Y., Sakurai H., Oketani K., Horie T., Yamoto C. and Moriga M. (1987) Effects of taurocholic acid/HCl alone or after pretreatment with geranylgeranylacetone on phospholipid metabolism in rat gastric mucosa. Biochem. Pharmacol. **36:** 4111–4117

9 Hirakawa T., Rokutan K. R., Nikawa T. and Kishi K. (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology **111:** 345–357

10 Tsuruma T., Yagihashi A., Koide S., Araya J., Tarumi K., Watanabe N. et al. (1999) Geranylgeranylacetone induces heat shock protein-73 in rat small intestine.Transplant. Proc. **31:** 572–573

11 Fudaba Y., Tashiro H., Ohdan H., Miyata Y., Shibata S., Shintal S. et al. (2000) Efficacy of HSP72 induction in rat liver by

orally administered geranylgeranylacetone. Transplant. Int. **13:** S278–281
12 Landry J., Bernier D., Chrétien P., Nicole L.M., Tanguay RM and Marceau N. (1982) Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res. **42:** 2457–2461
13 Li G. C. and Werb Z. (1982) Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc. Natl. Acad. Sci. USA **79:** 3218–3222
14 Subjeck J. R., Sciandra J. J. and Johnson R. J. (1982) Heat shock proteins and thermotolerance: a comparison of induction kinetics. Br. J. Radiol. **55:** 579–584
15 Lindquist S. and Craig E. A. (1988) The heat-shock proteins. Annu. Rev. Genet. **22:** 631–677
16 Mosser D. D., Caron A. W., Bourget L., Denis-Larose C. and Massie B. (1997) Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol. Cell Biol. **17:** 5317–27
17 Jaattela M., Wissing D., Kokholm K., Kallunki T. and Egeblad M. (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J. **17:** 6124–34
18 Pinot F., El Yaagoubi A. E., Christie P., Dinh-Xuan A. T. and Polla B. S. (1997) Induction of stress proteins by tobacco smoke in human monocytes: modulation by antioxidants. Cell Stress Chap. **2:** 156–161
19 Bachelet M., Mariéthoz E., Banzet N., Souil E., Pinot F., Polla C. Z. et al. (1998) Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. Cell Stress Chap. **3:** 168–176
20 Vayssier M., Banzet N., François D., Bellmann K. and Polla B. (1998). Tobacco smoke induces both apoptosis and necrosis in mammalian cells: differential effects of HSP70. Am. J. Physiol. **275:** L771–L779
21 Kroemer G., Dallaporta B. and Resche-Rignon M. (1998) The mitochondrial death: life regulator in apoptosis and necrosis. Annu. Rev. Physiol. **60:** 619–642
22 Banzet N., François D. and Polla B. S. (1999) Tobacco smoke induces mitochondrial depolarization along with cell death: effects of antioxidants. Redox Rep. **4:** 229–236
23 Trounce I., Byrne S. and Marzuki S. (1989) Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet **i:** 637–639
24 Smith R. A., Porteous C. M., Coulter C. V. and Murphy M. P. (1999) Selective targeting of an antioxidant to mitochondria. Eur. J. Biochem **263:** 709–716
25 Cossarizza A., Baccarani-Contri M., Kalachnikova G. and Franceschi C. A. (1998) A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1). Biochem. Biophys. Res. Commun. **30:** 40–45
26 Dubner D., Gisone P., Jaitovich I. and Perez M. (1995) Free radicals production and estimation of oxidative stress related to gamma irradiation. Biol. Trace Elem. Res. **47:** 265–270
27 Cossarizza A., Ceccarelli D. and Masini A. (1996) Functional heterogeneity of an isolated mitochondrial population revealed by cytofluorometric analysis at the single organelle level. Exp. Cell Res. **10:** 84–94
28 Polla B. S., Kantengwa S., François D., Salvioli S., Franceschi C., Marsac C. and Cossarizza A. (1996) Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. Proc. Natl. Acad. Sci. USA **25:** 6458–6463
29 Werninghaus K., Handjani R. M. and Gilchrest B. A. (1991) Protective effect of alpha-tocopherol in carrier liposomes on ultraviolet-mediated human epidermal cell damage in vitro. Photodermatol. Photoimmunol. Photomed. **8:** 236–242

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