Vol. 180, No. 1, 1991
October 15, 1991
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 308-314

FLAVONOIDS, BUT NOT PROTEIN KINASE C INHIBITORS, PREVENT STRESS PROTEIN SYNTHESIS DURING ERYTHROPHAGOCYTOSIS

Salomé Kantengwa and Barbara S. Polla

Allergy Unit, University Hospital, 1211 Geneva 4, Switzerland

Received August 16, 1991

Summary: Erythrophagocytosis induces in monocytes-macrophages the synthesis of stress proteins including the classical heat shock proteins (HSPs) and heme oxygenase (HO). To evaluate the role of oxygen radicals in this induction, we used the antioxidant flavonoids quercetin and kaempferol. These compounds inhibited HSP and HO synthesis, the latter being more sensitive. Quercetin and kaempferol also are inhibitors of protein kinase C (PKC). In order to determine whether inhibition of stress protein synthesis by flavonoids was mediated by their antioxidant properties or by PKC inhibition, we also tested more specific PKC antagonists, staurosporine and H-7. Staurosporine (SS) and H-7 decreased the synthesis of HSP70 and HSP83 but had no effect on HO. These data suggest that (1) erythrophagocytosis-related oxygen radicals are involved in the induction of the stress response in phagocytic cells, (2) the induction of HSPs and HO is differentially regulated, and (3) the effects of flavonoids on HO are linked to their scavenging activity rather than to PKC modulation.

The heat shock (HS) response has been observed in all organisms exposed to elevated temperatures. Since other agents also increase the synthesis of HS proteins (HSPs), these proteins are generally referred to as stress proteins. Whereas stress inducers elicit the increased expression of the same families of HSPs, there also are stress proteins unique to a particular agent(s). This is the case for the 32 kD stress protein associated with oxidative injury (1) and the glucose-regulated proteins induced by glucose deprivation (2).

HS gene activation involves the binding of a HS factor(s)to the HS element(s), this binding activity being heat-inducible (3). The cellular signals leading to the induction of stress protein synthesis include the presence of abnormal or degraded proteins (4). Several groups including our's have investigated the possibility that classical second messengers such as calcium or inositol phosphate participate in the cascade of stressful events leading to induction of HSPs (5, 6). The possibility that protein kinase C (PKC) is involved in HSP induction has recently been

Abbreviations: HS, heat shock; HSP, heat shock protein; HO, heme oxygenase; PKC, protein kinase C; SS, staurosporine; H-7, 1-(5-isoquinolinyl)-2-methylpiperazine; OFR, oxygen free radicals; O_2^-, superoxide; PMA, phorbol myristate acetate; oSRBC, opsonized sheep red blood cells; OZ, opsonized zymosan.

0006-291X/91 $1.50

suggested by the findings that HS increased the cytosolic PKC activity in rat embryo fibroblasts (7). It has also been suggested that HS-mediated induction of HSPs is directly related to the generation of reactive oxygen species and membrane lipid peroxidation (8); furthermore, oxygen derived toxic metabolites may also play a role in stress protein induction by leading to protein alterations (9, 10).

We have previously developed a model (erythrophagocytosis) in which induction of stress proteins appears directly dependent upon the generation of oxygen free radicals (OFR), and in particular, hydroxyl radicals (11). During erythrophagocytosis, the induction of the classical HSPs (47, 65, 70, 83-90, 110 kD) is associated with the synthesis of an oxidation-specific, non-heat-inducible stress protein, heme oxygenase (HO). HO is differentially regulated depending on the organism. Whereas the rat HO gene contains a HS concensus sequence and consequently is heat-activated (12, 13), heat fails to induce HO in mouse or human cells (12). In order to specify the respective roles of OFR and PKC in the induction of HSPs after HS and during erythrophagocytosis, we have used both flavonoids and the more specific PKC inhibitors, SS and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7). Flavonoids are radical scavengers, iron chelators (14), PKC inhibitors (15) and also prevent HSP induction after HS in HeLa cells (16). Here we report that the induction of stress proteins (and in particular HO) during erythrophagocytosis appears independent of PKC activation, and we provide further evidence suggesting reactive oxygen species play a role in stress protein induction.

Materials and Methods

Cells and culture methods. Peripheral human mononuclear cells were isolated by Ficoll gradient and monocytes were purified by adherence as previously described (17). Freshly isolated cells were cultured in antibiotic-free RPMI 1640 medium (Gibco Laboratories, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% glutamine (Gibco), and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Superoxide measurement. Monocytes were washed three times with phosphate-buffered saline (PBS; Gibco) and resuspended in a buffer containing 138 mM NaCl; 6 mM KCl; 1 mM $MgSO_4.7H_2O$; 1.1 mM $CaCl_2.2H_2O$; 0.2 mM EGTA; 5.5 mM glucose; 20 mM Hepes, pH 7.4. 10^6 cells were stimulated with 1.6×10^{-7} M phorbol myristate (PMA; Sigma) or 3 mg/ml opsonized zymosan (Sigma) for 30 min at 37°C and superoxide (O_2^-) measured by the superoxide dismutase-inhibitable reduction of cytochrome *c* as previously described (18). The effects of SS (5-100 nM) and H-7 (10-100 µM) were tested by preincubating the cells for 15 min with the inhibitors before stimulation with PMA or OZ.

Erythrophagocytosis. Sheep red blood cells (SRBC; BioMérieux, France) were washed three times by centrifugation with physiologic NaCl and opsonized with rabbit anti-SRBC in 1% bovine serum albumine (BSA)-NaCl for 30 min. Following opsonization, erythrocytes were washed again and added to culture dishes at a ratio of SRBC to phagocytes of 600:1. The cells were allowed to phagocytose for 3 h and then labeled for 90 min as described below. Percentage phagocytic cells were determined by counting 200 cells in each of 3 different cellular prepartions stained with May-Grünwald-Giemsa.

Exposure to HS and electrophoresis. Cells were exposed to 44°C in a water bath for 20 min in 25 mM Hepes-buffered medium. SS (50 nM) or H-7 (100 µM) were added 15 min before, and the flavonoids quercetin or kaempferol (100 µM) 5 min before HS or the beginning of erythrophagocytosis. The cells were allowed to recover at 37°C for 2 h after HS then labeled with 9 µCi/ml [^{35}S]methionine (Amersham, Buckinghamshire, UK) for 90 min. The cells were harvested, washed twice with PBS, lysed in sodium dodecyl sulfate (SDS) buffer (19) and

boiled for 10 min in the presence of 4% b-mercaptoethanol. Extracts corresponding to equal cell numbers were electrophoresed on SDS-polyacrylamide slab gels according to Laemmli (19). The proteins were detected by autoradiography on X-Omat AR films (Kodak, Lausanne, Switzerland). Scanning densitometry was performed with a Genoscan TM laser beam densitometer (Genofit, Geneva, Switzerland).

Results

Inhibition of the stress response by flavonoids. Following addition of quercetin or kaempferol, monocytes were exposed to 44°C for 20 min and allowed to recover from HS at 37°C for 2 h, while parallel cultures were used for phagocytosis of oSRBC for 3 h. The pattern of protein synthesis following these treatments is shown in Fig. 1a, and the corresponding scannings in Fig. 1b. First, the flavonoids had no effect on protein synthesis in unstressed cells (Fig 1, a and b, lanes 2 and 3). Second, as previously described (16) both quercetin and kaempferol decreased the heat-induced synthesis of HSP70 and HSP83 (Fig 1, a and b, lanes 5 and 6). Third, during erythrophagocytosis, the flavonoids completely inhibited the induction of the 32 kD oxidative stress-specific protein related to erythrophagocytosis (Fig 1, a and b, lanes 8 and 9) but only decreased the synthesis of the classical HSPs (70 and 83 kD). Kaempferol, which was the more potent inhibitor of stress protein synthesis, had no effect on phagocytosis (97% ±

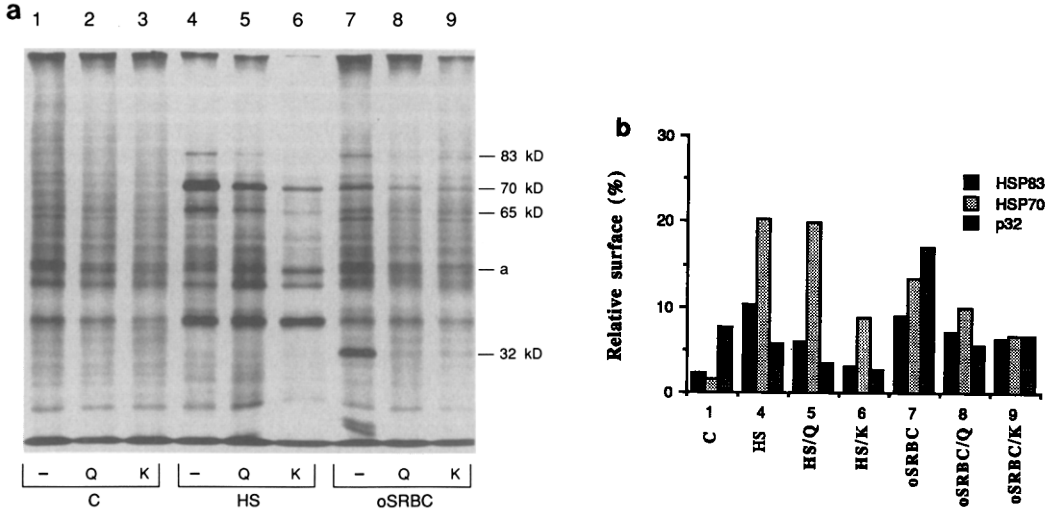

Fig. 1. (a) Inhibition of stress response in human monocytes by quercetin (Q) or kaempferol (K). Human monocytes were preincubated with the flavonoids before exposure to heat or erythrophagocytosis, and cells labeled and processed as described under Materials and Methods. Aliquots corresponding to equal cell numbers were loaded on each lane. Quercetin and kaempferol had no effect by themselves on protein synthesis by monocytes: *lane 2* (Q), *lane 3* (K) vs *lane 1*; in contrast, HSP70 and HSP83 synthesis was decreased in the presence of flavonoids: *lane 5* (Q) and *lane 6* (K) vs *lane 4*; erythrophagocytosis-related 32 kD protein synthesis was inhibited by the flavonoids: *lane 8* (Q), *lane 9* (K) vs *lane 7*.
(b) Digrammatic representation of relative surface of band intensity obtained by scanning densitometry. The numbers of the lanes correspond to Fig. 1a. In the presence of the flavonoids, the levels of HO are similar to control levels (lanes 8 and 9 vs lane 1).

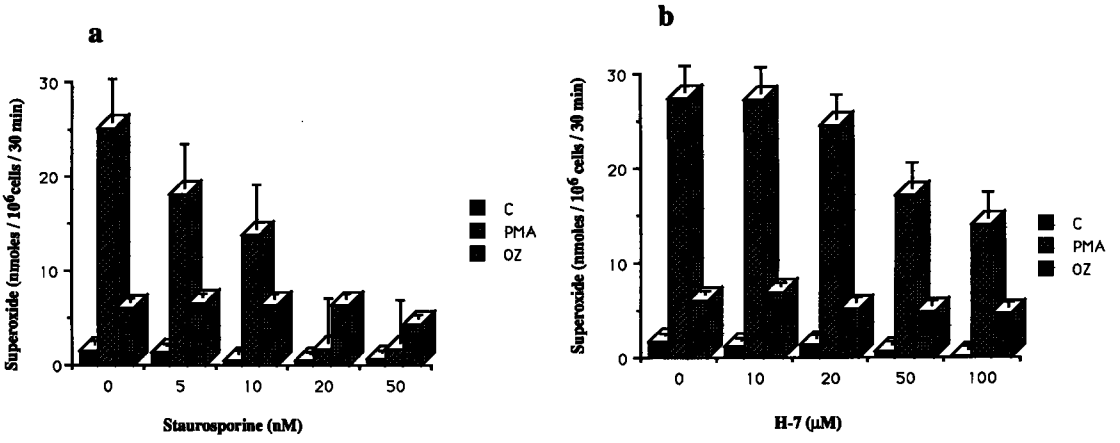

Fig. 2.(a-b)Effect of protein kinase inhibitors on PMA- or opsonized zymosan-stimulated superoxide production by monocytes. The cells were preincubated 15 min with SS (*a*) or H-7 (*b*) and then stimulated for 30 min at 37°C with either 1.6 x 10^{-7} M PMA or 3 mg/ml OZ. The results represent means ± SEM from 3 different experiments in which each sample was performed in triplicates. PMA-stimulated superoxide production was inhibited in a dose-dependent manner by both PKC antagonists. As expected superoxide production stimulated by OZ was not affected by PKC inhibitors.

0.2 phagocytic cells [mean ± SEM, n=3] as compared to control), whereas quercetin reduced the number of phagocytic cells by 32% ± 2.7 (mean ± SEM, n=3).

Effects of PKC inhibitors on HSP synthesis. The inhibitory effects of SS (5-100 nM) or H-7 (10-100 μM) on PKC activity were first established in our system on the PMA-stimulated generation of O_2^- as a PKC-mediated pathway. Monocytes were incubated with increasing concentrations of PKC inhibitors for 15 min before the 30 min stimulation with either 1.6×10^{-7} M PMA or OZ (3 mg/ml), a phagocytic stimulus. Fig. 2a shows the results from 3 distinct experiments on the effects of SS. SS inhibited PMA-stimulated O_2^- production in a dose-dependent manner (from 30 to 6 nmoles with increasing SS from 0 to 50 nM). H-7 had similar effects but to a lesser extent (Fig. 2b). Neither SS nor H-7 affected OZ-mediated O_2^- generation because OZ activates O_2^- production through a phospholipase A2-mediated pathway (18). Having established that SS and H-7 inhibit PKC-mediated O_2^- production in monocytes, we used the same inhibitors to determine whether a PKC-mediated event was implicated in the stress response induction. Using optimal doses of SS (50 nM) and H-7 (100 μM) we evaluated their effects on the synthesis of HSPs in cells exposed to 44°C or to opsonized SRBC (Fig. 3). Whereas SS and H-7 slightly inhibited the heat-induced HSP synthesis, they had no significant effect on stress protein synthesis during erythrophagocytosis (Fig. 3).

Discussion

The stress/HS response results in the expression of a highly conserved set of genes, the HS genes and in the synthesis of HSPs (20), following exposure to a variety of stressful

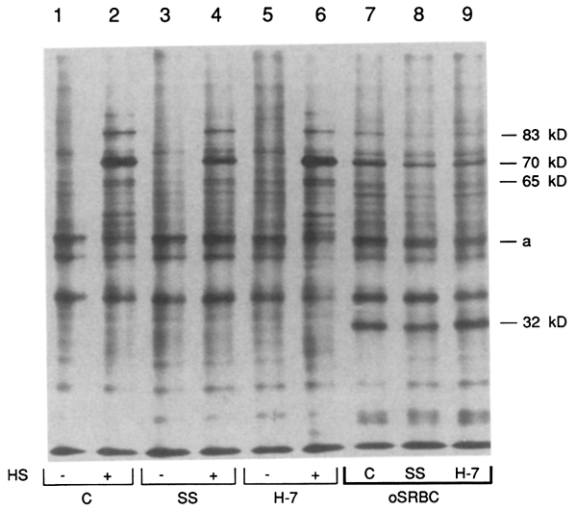

Fig. 3. Effect of SS or H-7 on HSP synthesis in human monocytes. Monocytes were treated with the inhibitors for 15 min and then exposed (+) or not (-) to 44°C or to oSRBC in the presence or the absence of SS (SS, 50 nM) or H-7 (100 μM). The more effective PKC inhibitor SS (see Fig 2a) decreased HSP synthesis after HS (*lane 4 vs lane 2*), whereas SS or H-7 had no effect on HO induction during erythrophagocytosis (*lanes 8* and *9* vs *lane 7*).

conditions. We have previously shown that erythrophagocytosis induced in human monocytes-macrophages the synthesis of classical HSPs as well as HO (11). We hypothesized that HSP induction under these conditions was mediated by the iron-dependent O_2^--driven Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO$), which results in the generation of reactive oxygen species. To test this hypothesis, we first examined the effects of antioxidant molecules such as flavonoids. Flavonoids have been previously shown to prevent HSP synthesis after HS in HeLa cells and have been suggested to act at the transcriptional level (16). Flavonoids are non-toxic plant phenolic compounds with antioxidant properties, and provide direct scavenging activity by preventing the O_2^--related Fenton reaction and limiting lipid peroxidation (14). In human monocytes, quercetin and kaempferol inhibited both the heat- and the erythrophagocytosis-induced stress response (Fig. 1). The inhibitory effects of kaempferol on stress protein induction during erythrophagocytosis were not related to a decreased phagocytosis. These effects were most striking on the induction of the erythrophagocytosis-related HO, which became undetectable when erythrophagocytosis occured in the presence of flavonoids. Since HO is considered as an oxidant-specific stress proteins, we suggest that the inhibitory effect of flavonoids on HO is related to their antioxidant properties. On the other hand, the partial inhibition, by flavonoids, of heat-induced HSPs supports Burdon's hypothesis of a role for reactive oxygen species in HSP induction after HS (8).

Another target of flavonoids is the ubiquitous transmembrane enzyme PKC (15). Since the PKC activity is increased during HS, flavonoids could also interfere with HSP synthesis by

inhibiting PKC pathway(s). Quercetin and kaempferol have potent PKC inhibition activity (15). We thus tested in our model the more specific PKC inhibitors SS and H-7. SS inhibits phorbol ester-induced pathways, such as a rise in intracellular calcium (21) and CR3-mediated phagocytosis in neutrophils (22); H-7 is less potent than SS, acting at micromolar concentrations and showing limited selectivity (23).

We first established that these compounds selectively inhibited a PKC-mediated event in our model (i.e., involvement of PKC in the activation of NADPH oxidase has been described by Maridonneau-Parini et al. (18). In phagocytic cells, NADPH oxidase is a key enzyme which catalyzes the formation of O_2^-; other reactive oxygen species (hydrogen peroxide and, in the presence of iron, hydroxyl radicals) are then generated from O_2^-. NADPH oxidase can be activated either through a PKC pathway (after stimulation with PMA) or a phospholipase A2 pathway (after stimulation with opsonized zymosan). Both SS and H-7 inhibited PMA- but not zymosan-stimulated superoxide production by monocytes (Fig. 2, a and b) in a dose-dependent manner, stauropsorine being more effective than H-7. When tested at optimal concentrations, SS and H-7 slightly decreased heat-induced HSP synthesis but had no effect on the erythrophagocytosis-related induction of HO (Fig. 3). Thus, whereas induction of the classical HSPs appears, at least in part, dependent on PKC, HO induction, in contrast, appears strictly dependent upon oxygen reactive species.

In conclusion, our results indicate that PKC is not involved in the inhibition of erythrophagocytosis-mediated HO synthesis by flavonoids, and further support the role for free radicals in the induction of stress proteins in phagocytes. Different mechanisms appear to regulate the heat- or erythrophagocytosis-induced stress response, both a PKC-mediated event and OFR being involved in heat-induced HSP synthesis, whereas OFR would represent the main stressor during erythrophagocytosis.

Acknowledgments: This work was supported by the Swiss National Research Foundation grant no. 32-028645.90 to BSP. We are grateful to Aileen Healy for helpul comments and critical review and to Florence Villars for skillful technical assistance.

References

1. Keyse, S.M., and Tyrrell, R.M. (1987) J. Biol. Chem. 262, 148221-14825.
2. Welch, W.F., Garrels, J.G., Thomas, G.P., Lin, J.J., and Feramisco, J.R. (1983) J. Biol. Chem. 258, 7102-7111.
3. Zimarino, V., and Wu, C. (1987) Nature 327, 727-730.
4. Ananthan, T., Goldberg, A.L., and Voellmy, R. (1986) Science 2232, 522-524.
5. Stevenson, M.A., Calderwood, S.K., and Hahn, G.M. (1987) Cancer Res. 47, 3712-3717.
6. Kantengwa, S., Capponi, A.M., Bonventre, J.V., and Polla, B.S. (1990) Am. J. Physiol. 259 (Cell Physiol. 28), C77-C83.

7. Wooten, M.W. (1991) Exp. Cell. Res. 193, 274-278.
8. Burdon, R.H., Gill, V.M., and Rice-Evans, C. (1987) Free Rad. Res. Commun. 3, 129-139.
9. Oliver, C.N., Sarke-Reed, P.E., Stadtman, E.R., Lin, G.J., Carney, J.M., and Floyd, R.A. (1990) Proc. Natl. Acad. sci. USA 87, 5144-5147.
10. Schraufstätter, I., Hyyslop, P.A., Jackson, J.H., and Cochrane, C.G. (1988) J. Clin. Invest. 82, 1040-1050.
11. Clerget, M., and Polla, B.S. (1989) Proc. Natl. Acad. Sci. USA 87, 1081-1085.
12. Taketani, S., Hohno, H., Yoshin aga, T., Tokunaga, R. (1988) Biochem. Intern. 17, 665-672.
13. Shibahara, S., Muller, R.M., and Taguchi, H. (1987) J. Biol. Chem. 262, 12889-12892.
14. Afanas'Ev, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., and Potapovitch, A.I. (1989) Biochem. Pharmacol. 38, 1763-1769.
15. Farriola, P.C., Cody, V., and Middleton, E. Jr. (1989) Biochem. Pharmacol. 38, 1617-1624.
16. Hosokawa, N, Hirayoshi, K., Nakai, A., Hosokawa, Y., Marui, N., Yoshida, M.,Sakai, T., Nishino, H., Aoike, A., Kawai, K., and Nagata, K. (1990) Cell Struct. Funct. 15, 393-401.
17. Polla, B.S., Healy, A.M., Wojno, W.C., and Krane, S.M. (1987) Am. J. Physiol. 252, C640-C649.
18. Maridonneau-Parini, I., Tringale, J.N., and Tauber, A.I. (1986) J. Immunol. 137, 2925-2929.
19. Laemmli. U.K. (1970) Nature 227, 680-685.
20. Lindquist, S., and Craig, E.A. (1988) Annu. Rev. Genet. 22, 631-677.
21. Erne, P., Schachter, M., Fabbro, D., Miles, C.M.M., and Sever, P.S. (1987) Biochem. Biophys. Res. Commun. 145, 66-72.
22. Roubey, R.A.S, Ross, G.D., Merril, J.T., Walton, F., Reed, W., Winchester, R.J., and Buyon, J.P. (1991) J. Immunol. 146, 3557-3562.
23. Hidaka, H., Inagami, M., Kawamoto, S., and Sasaki, Y. (1984) Biochem. 23, 5036-5041.