Effects of tobacco smoke and benzo[a]pyrene on human endothelial cell and monocyte stress responses

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Effects of tobacco smoke and benzo[a]pyrene on human endothelial cell and monocyte stress responses. Am J Physiol Heart Circ Physiol 280: H1293–H1300, 2001.—Smoking is an important risk factor for atherosclerosis. We compared tobacco smoke filtrate with benzo[a]pyrene (a prominent xenobiotic component of tobacco smoke) for the capacity to induce stress proteins and cause cell death in human monocytes and vascular endothelial cells, two cell types that are involved in the formation of atherosclerotic lesions. Exposure to freshly prepared filtrates of tobacco smoke induced in both monocytes and endothelial cells expression of the inducible heat shock protein (HSP)70 and heme oxygenase-1 (HO-1) and produced loss of mitochondrial membrane potential. Later, cell death by apoptosis or necrosis occurred depending on the concentration of tobacco smoke. These toxic effects could be prevented by the antioxidant N-acetylcysteine. In contrast, exposure of these cells to benzo[a]pyrene alone evoked neither stress proteins nor mitochondrial damage but did induce cell death by necrosis. Thus our results indicate that tobacco smoke rapidly induces complex oxidant-mediated stress responses in both vascular endothelial cells and circulating monocytes that are independent of the benzo[a]pyrene content of the smoke.

Cigarette smoking is a risk factor for atherosclerotic cardiovascular disease, pulmonary emphysema, and cancer (16, 25). Among the toxic compounds contained in tobacco smoke (TS) are reactive oxygen species (ROS), polycyclic aromatic hydrocarbons such as benzo[a]pyrene (BaP), cadmium, and nicotine (4, 8, 18, 33). In animal models BaP alone induces atherosclerotic lesions (34). Exposure to TS produces cellular injury but also induces cytoprotective stress proteins, including heat shock proteins (HSPs) and heme oxygenase-1 (HO-1). HSPs are induced by a variety of injurious stimuli including ROS (19, 29, 32), and HSP expression is altered in many pathological conditions including atherosclerosis. Activation of blood monocytes and the interaction of monocytes with vascular endothelium are important in early stages of atherosclerosis. We had reported that in vitro exposure to TS induces in human monocytes and in the premonocytic line U-937 the synthesis of HSP and in particular the cytosolic, inducible, highly protective 72-kDa protein HSP70 (29, 39). Under certain conditions increased generation of ROS within established atherosclerotic lesions may also lead to the synthesis of HSPs (36). It is not yet clear whether HSP70 has a role in early atherosclerosis, but considering the ubiquitous protective functions of HSP70, its increased expression could represent a protective mechanism against vascular injury. Besides HSPs other proteins may be induced in vascular cells and monocytes as adaptive mechanisms. In particular TS also induces the oxidation-specific stress protein HO-1 in monocytes (29). HO-1 catalyzes the oxidative degradation of heme to biliverdin, which in turn is reduced to bilirubin, an antioxidant. This mechanism along with the induction of ferritin mediates the cytoprotective antioxidant effects of HO-1 (42).

Because TS has been implicated in the initiation and progression of atherosclerotic vascular lesions, whereas exposure to BaP alone has resulted in atherosclerosis in experimental models, we compared the effects of TS and BaP on the induction of stress proteins and cell death in human monocytes and endothelial cells, two cell types that are closely involved in the formation of atherosclerotic plaques. Endothelial dysfunction is an early hallmark of atherosclerosis, so that effects of tobacco components on endothelium are important to determine. We have recently reported that despite inducing HSP, TS exposure results in cell death of monocytes either by apoptosis (programmed cell death) or by necrosis, depending on the TS concentration (39). It is now generally recognized that mitochondria play a key role in ROS-mediated cell death and in the cellular “choice” between apoptosis and necrosis (31, 35, 39).
Disruption of the mitochondrial membrane potential (ΔΨ) is generally considered as an early and prerequisite step in the pathways leading to ROS-mediated apoptosis (32, 46). Thus we also compared the effects of TS and BaP on ΔΨ in human monocytes and endothelial cells. Finally, given the relevance of ROS in TS-mediated toxicity and atherogenesis, we investigated the effects of the glutathione (GSH)-restoring antioxidant N-acetylcysteine (NAC) on TS-induced stress responses in these cells.

METHODS

Reagents and Antibodies

Paraformaldehyde, saponin, BaP, gelatin, NAC, and glucose were purchased from Sigma (St. Louis, MO). Culture media (RPMI 1640 and DMEM), FCS, PBS, trypsin-EDTA (0.05% trypsin-0.02% EDTA), L-glutamine, BSA (fraction V), HEPES buffer, and penicillin-streptomycin were from ICN Biochemicals (Costa Mesa, CA). 5,5′,6,6′-Tetrachloro-1,3,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). Annexin V (AV) conjugated to FITC (AV-FITC) and propidium iodide (PI) were from Boehringer (Mannheim, Germany). Monoclonal antibodies against the inducible form of HSP70 (mouse IgG1, SPA-110) or HO-1 (mouse IgG1, OSA-110) were from Stressgen (Victoria, Canada). The F(ab′)2 fragment of rabbit anti-mouse IgG conjugated to FITC (used as secondary antibody) was from Dako (Glostrup, Denmark).

Cells and Culture Conditions

Human peripheral blood mononuclear cells from healthy volunteers were isolated by Ficoll gradient centrifugation and purified by adherence as previously described (17). Monocytes (2.5 × 10⁶ cells/ml) were maintained in RPMI 1640 medium containing 10% FCS, 2 mmol/l glutamine, and 25 mmol/l HEPES. To ensure a uniform population of endothelial cells, we utilized a stable endothelial cell line (transfected human bone marrow endothelial cells, TrHBMECs), derived from normal human microvascular endothelial cells, that has been characterized as maintaining a phenotype typical of normal primary endothelial cells of both macrovascular and microvascular origin (37). TrHBMECs were cultured on 0.2% gelatin in DMEM containing 5% FCS, 10 mmol/l HEPES, 0.1% glucose, 3 mmol/l glutamine, and 120 µg/ml penicillin-streptomycin. In all experiments, TrHBMECs and human monocytes were placed in fresh medium containing 0.5% FCS before exposure to TS or BaP.

Exposure to Stress

A peristaltic pump smoke machine (Heinrich Borgwaldt RM1/G, Hamburg, Germany) was used to generate TS-bubbled PBS solution from mainstream smoke of standard cigarettes (reference cigarette 2R1, University of Kentucky) generated through a puffing mechanism mimicking a standardized human smoking pattern (duration 2 s/puff; frequency 1 puff/min; volume 35 ml/puff). The aqueous smoke fractions from one cigarette correspond to 10 puffs (350 ml) of smoke bubbled through 5 ml PBS. This was termed TS. The final dilutions of TS in the cell culture medium are expressed in puffs per milliliter. After extensive dose-response experiments (29, 39), we used concentrations between 0.03 and 0.24 puffs/ml in the experiments reported here.

We exposed cells to concentrations of BaP similar to those generally reported in the experimental literature, i.e., 1, 5, and 10 µmol/l. These concentrations are even higher than the concentrations of BaP present in TS, because one cigarette contains on average 30 ng of BaP (18), 100% of which passes into the smoke. Thus the concentration of BaP present in TS, extrapolated from the molarity of BaP present, is about 0.02 µmol/l. BaP was dissolved in DMSO at 50 mmol/l and diluted to the desired final concentrations in culture medium, and control cells were incubated with a comparable concentration of DMSO alone.

For testing the effects of NAC, cells were pretreated for 1 h with 25 mmol/l of the drug, which was kept in the medium for the entire time of TS exposure (4 or 16 h). In preliminary studies this concentration was determined to be nontoxic.

HSP70 and HO-1 Determination by Flow Cytometry

Monocytes in suspension (10⁶ cells/condition) and endothelial cells at confluence in 35-mm wells were exposed to TS or BaP for 4 h before analysis for induction of HSP70 or HO-1 expression. We used flow cytometry as a sensitive, quantitative, and reproducible method for analyzing expression of intracellular stress proteins (2). Briefly, 1 × 10⁶ monocytes or endothelial cells (the latter detached from culture dishes by brief trypsinization) were washed twice with PBS before being fixed for 10 min in 3% paraformaldehyde. After fixation the cells were resuspended for 10 min in 50 µl of 0.6% saponin and 50 µl of anti-HSP70 or anti-HO-1 antibodies prediluted to a 1:100 ratio in PBS with 1% BSA (PBS-BSA). After being washed twice in PBS-BSA, the cells were resuspended in 100 µl of secondary antibody [FITC-conjugated F(ab′)2 fragment of rabbit anti-mouse IgG] diluted to a 1:30 ratio in PBS-BSA. Cells were washed to remove unbound FITC, resuspended in 0.5 ml of PBS-BSA per sample, and kept in the dark at 4°C until analysis. Flow cytometry was performed on 5,000 cells/sample using an EPICS Elite flow cytometer (Coulter, Miami, FL) equipped with a single 488-nm argon laser. For each sample the induction of HSP70 or HO-1 expression was calculated as the ratio of cells expressing HSP70 or HO-1 after TS or BaP exposure compared with cells expressing these proteins in control conditions.

Determination of ΔΨ

ΔΨ was analyzed by JC-1 fluorescence as previously described (10, 11). The lipophilic cation JC-1 forms J-aggregates in the matrix of intact mitochondria (emitting at 590 nm) or is released in a monomeric form (527 nm) from depolarized mitochondria. Thus mitochondrial membrane depolarization is associated with a shift in JC-1 fluorescence emission from red to green. Cells were incubated for 10 min in the dark with 0.5 ml of JC-1 (20 mg/ml in PBS), washed, and suspended in 0.5 ml of PBS for fluorescent activated cell sorting (FACS) analysis. We counted 5,000 cells/sample in acquisition and analyzed them using Elite 4.01 software. The results were expressed as the percentage of cells with disrupted mitochondrial membranes.

Flow Cytometric Analysis of Cell Death

Phosphatidylserine externalization at the plasma membrane occurs as an early and universal event in apoptotic cells, although membrane integrity is preserved (12). Surface exposure of phosphatidylserine was monitored to assess apoptosis, using the high-affinity binding of AV for negatively
charged phospholipids. In contrast, plasma membrane disruption (a characteristic of necrosis) was monitored by flow cytometry using cellular uptake of PI. After 16 h of treatment with vehicle, BaP, or TS, cells (10^6 cells/condition) were incubated in HEPES buffer in the presence of both AV-FITC and PI as described by the manufacturer and then analyzed by flow cytometry. After excitation, the red emission of PI (at 617 nm) was analyzed in FL1 and the green emission of FITC (at 525 nm) was analyzed in FL2. Double labeling of cells with a combination of fluoresceinated AV and PI allows the detection and distinction of intact living (AV−, PI−), apoptotic (AV+, PI−), or necrotic (AV+/-, PI+) cells. Cells in each of these categories were expressed as the percentage of total cells counted.

**Fig. 1.** Effects of tobacco smoke (TS) and benzo[a]pyrene (BaP) on heat shock protein 70 (HSP70) expression of monocytes and endothelial cells. Cells were exposed to TS doses ranging from 0.06 to 0.24 puffs/ml (A) or to BaP at concentrations from 1 to 10 μmol/l (B) for 4 h. Cells were then labeled with anti-human HSP70. Analysis of immunofluorescence detected with rabbit anti-mouse FITC was performed by flow cytometry. Values are means ± SE of HSP70-expressing cells relative to controls [(percent HSP70-expressing cells after treatment)/(percent basal HSP70-expressing cells)]; n = 4 experiments. *P < 0.05 and **P < 0.01 treated vs. control.

**Fig. 2.** Effects of TS and BaP on heme oxygenase-1 (HO-1) expression of monocytes and endothelial cells. Cells were exposed to TS from 0.03 to 0.24 puffs/ml (A) or to BaP at concentrations from 1 to 10 μmol/l (B) for 4 h before being labeled with anti-human HO-1. Analysis of immunofluorescence detected with rabbit anti-mouse FITC was performed by flow cytometry. Values are means ± SE of HO-1-expressing cells relative to controls [(percent HO-1-expressing cells after treatment)/(percent basal HO-1-expressing cells)]; n = 5 experiments. **P < 0.01 treated vs. control.

**Statistical Analysis**

Data are presented as means ± SE. Student's t-test was used for the comparison of two groups of data.

**RESULTS**

**Effects of TS and BaP on Expression of Stress Proteins in Monocytes and Endothelial Cells**

**HSP70.** With the use of biometabolic labeling and Western blotting, we previously reported that TS in-
duces HSPs in human monocyte populations (29). Now with the more sensitive flow-cytometric detection of intracellular HSP70 using specific monoclonal antibodies in permeabilized cells (2), we compared the effects of TS and BaP on HSP induction in both monocytes and endothelial cells. Cells were exposed to TS for 4 h at concentrations ranging from 0.03 to 0.24 puffs/ml. At this time of exposure, 100% of cells were viable (data not shown). Results are given as the percentage of treated cells expressing HSP70 compared with controls. As previously described, human monocytes showed great variability in basal HSP70 expression (2), whereas basal expression of HSP70 in TrHBMECs was low and varied minimally in different experiments (2.1 ± 0.2% for four experiments). TS (Fig. 1A) induced a dose-dependent increase in HSP70 expression within 4 h in both monocytes and endothelial cells, although BaP (Fig. 1B) did not induce HSP70 in either cell type even when cells were exposed to BaP for ≥16 h (not shown).

HO-1. Experimental conditions for assessing the induction of HO-1 by TS or BaP were similar to those used to test the induction of HSP70. Results are presented as the percentage of cells expressing HO-1 relative to controls. Variability of basal expression of HO-1 was again higher in monocytes and lower in endothelial cells (12.4 ± 1%, n = 3). Low concentrations of TS (0.03 and 0.06 puffs/ml) induced HO-1 similarly in both monocytes and endothelial cells (Fig. 2A). At higher concentrations of TS, HO-1 expression diminished in both cell types and became undetectable after exposure to concentrations ≥0.12 puffs/ml, although no signs of cell death were observed at 4 h under these experimental conditions (data not shown). In contrast, BaP failed to alter HO-1 expression in monocytes or in endothelial cells after 4 h (Fig. 2B) or 16 h of exposure (data not shown).

Effects of TS and BaP on Δψ of Monocytes and Endothelial Cells

Several types of cellular stresses including ROS lead to disruption of Δψ, which is generally considered as an early step toward cell death. We compared the effects of TS (Fig. 3A) and BaP (Fig. 3B) on Δψ in human monocytes and endothelial cells after 4 h of cell exposure to these substances. One representative example of the effects of both TS and BaP on Δψ in both cell types is shown in Fig. 3. TS induced a
dose-dependent disruption in Δψ (Fig. 3A), whereas BaP had no effect in either cell type (Fig. 3B).

**Effects of TS and BaP on Cell Death in Monocytes and Endothelial Cells**

Apoptosis (Fig. 4, A and C) and necrosis (Fig. 4, B and D) were analyzed by AV-FITC binding and PI uptake, respectively, after 16 h of exposure to TS or BaP. This time period was selected after extensive time-course experiments, as we had previously reported that TS-mediated cell death is not detected before 16 h of exposure (39). Both monocytes and endothelial cells exposed to low concentrations of TS (0.03 and 0.06 puffs/ml) for this period underwent cell death by apoptosis (Fig. 4A). For concentrations above 0.12 puffs/ml, the apoptotic population decreased, although there was a steady increase in cell death by necrosis (Fig. 4B), confirming previous studies on other mammalian cells (41). In contrast, BaP did not induce any detectable apoptosis (Fig. 4C), although at ≥ 5 μmol/l, BaP induced necrosis in both monocytes and endothelial cells (Fig. 4D).

**Effect of NAC Pretreatment on Stress Protein Induction**

Because we had previously shown that exposure of monocytes to the oxidant H₂O₂ induced expression of stress proteins, disrupted Δψ, and produced cell death by apoptosis at low H₂O₂ concentrations and by necrosis at higher H₂O₂ concentrations (20, 22, 27), we tested whether the antioxidant NAC could protect monocytes and endothelial cells from the toxic effects of TS. Pretreatment of both cell types for 1 h with NAC before introduction of TS completely abolished TS-mediated induction of stress protein expression (Fig. 5) and blocked cell death by apoptosis and necrosis. These cytoprotective effects of NAC were of similar degree in endothelial cells (Table 1) and in monocytes (3). These results support a role for ROS as mediators of TS-induced stress responses and cell death in endothelial cells as well as in monocytes.

**DISCUSSION**

When human endothelial cells were exposed to aqueous filtrates of TS in vitro, we observed that stress proteins such as HSP70 and HO-1 were rapidly induced in parallel to mitochondrial membrane depolarization, the latter representing an early step in the apoptotic pathway toward cell death. These responses reflected the concentration of TS to which the cells were exposed. To our knowledge this is the first report that exposure to TS induces a stress response in endothelial cells. The stress responses of endothelial cells to TS closely resembled those that we previously reported for peripheral blood monocytes. Upon studying monocytes and endothelial cells in parallel within the same experiments, we determined that similar concentrations of TS produced similar oxidant-dependent responses in both cell types. In contrast to the effects of TS, BaP, a xenobiotic present in TS that has been shown to induce atherosclerosis in experimental models, did not induce HSP70 or HO-1 expression nor did it produce Δψ or subsequent induction of apoptosis in our in vitro study even when used at concentrations considerably higher than those found in TS.

The induction of both HSP70 and HO-1 in cells has been observed under a variety of conditions characterized by oxidative stress (19, 30). The regulation of
HSP70 expression by TS was different from that of HO-1: HO-1 was induced by low concentrations of TS, whereas HSP70 was induced by higher concentrations of TS that decreased HO-1 expression. TS regulation of HSP70 expression is mediated by the activation of the heat shock factor (HSF) and high concentrations of TS is associated with a rapid inhibition of nuclear factor-κB (NF-κB) (40). The fact that both HSF and NF-κB are involved in regulation of HO-1 (24) might explain the observed differences; indeed, for high TS concentrations the inhibition of NF-κB may prevent HO-1 upregulation.

In our model TS induced HSP70 and HO-1 to a similar extent in both monocytes and endothelial cells. However, the response of endothelial cells to oxidative stress had been reported to be more limited than that of monocytes (22). It has been suggested that monocytes contribute to endothelial cell protection by the transfer of their own HSPs to endothelial cells (5) in a manner similar to the transfer from glia to axon described by Brown (6) in the brain. Our results indicate that endothelial cells are able to produce protective stress protein molecules in amounts similar to monocytes and with a similar time course, at least when exposed to TS. Thus a strong induction of HSP70 in endothelial cells might be a specific response to TS. Considering the functions of molecular chaperones, TS-induced stress proteins might play a role in the cellular cross talk between monocytes and endothelial cells that modulates the progression of the atherosclerotic plaque.

Expression of HSP70 and HO-1 is higher in atherosclerotic lesions than in normal vascular tissue and even early plaque formation is associated with changes in the distribution of vascular HSPs and HO-1 (5, 21, 44). Although HO-1 expression in TS-exposed cells did not protect the cells from death, HO-1 may nevertheless protect against the induction of an inflammatory response in the vascular wall because HO-1 induction results in decreased monocyte chemotaxis in response to low-density lipoprotein oxidation (17). Similarly, HSP70 expression by endothelial cells could exert cytoprotective functions as well as reflect stress exposure. However, our findings indicate that HSP70 expression in response to TS fails to protect endothelial cells from cell death with prolonged TS exposure because, even though HSP70 is expressed early, cells will finally die either by apoptosis or necrosis, depending on TS concentration. Interestingly, the induction of HSP70 as an early biomarker of apoptosis has been observed in other apoptotic models (7, 14), suggesting that in some models HSP70 induction is not sufficient to prevent apoptosis. Moreover, we have previously shown that HSP70 overexpression in rat pancreatic cells protected those cells from TS-induced necrosis.

Table 1. Effects of NAC pretreatment on TS-mediated cell death

<table>
<thead>
<tr>
<th>Tobacco Smoke, puffs/ml</th>
<th>−NAC</th>
<th>+NAC</th>
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<tbody>
<tr>
<td>0.06</td>
<td>Apoptosis</td>
<td>64.8 ± 2.5</td>
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<tr>
<td></td>
<td>Necrosis</td>
<td>5.2 ± 1.5</td>
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<tr>
<td>0.12</td>
<td>Apoptosis</td>
<td>64.8 ± 5.2</td>
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<tr>
<td></td>
<td>Necrosis</td>
<td>29.1 ± 2.4</td>
</tr>
<tr>
<td>0.24</td>
<td>Apoptosis</td>
<td>64.8 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
<td>64.8 ± 6.1</td>
</tr>
</tbody>
</table>

Values are means ± SE of percent apoptotic or necrotic cells; n = 3 experiments. Cells were preincubated with 25 mmol/l N-acetyl-D-cysteine (NAC) for 1 h before exposure to tobacco smoke (TS).
The protected cells died instead by apoptosis (39). This indicates that HSP70 overexpression could promote a change in the type of cell death rather than resistance per se to TS toxicity (39). An important consequence of changing the mode of cell death from necrosis to apoptosis at the vascular level would be to decrease the inflammatory process that accompanies cell death by necrosis but is absent when cells die by apoptosis. This change would favor normal vascular remodeling, whereas an inflammatory process favors atherogenesis.

In parallel to TS-mediated induction of stress proteins in these studies, TS also altered mitochondria by disrupting Δψ, an early event in programmed cell death that could contribute to TS-mediated pathology. Δψ disruption may also be perceived as a novel biomarker for cellular responses to exposure to TS or related oxidants (23), whereas it is not produced by exposure of cells to BaP. Indeed, our results suggest that BaP can be directly cytotoxic without producing a stress response, disrupting Δψ, or initiating apoptosis. BaP has been considered as a classic toxic component of TS, and BaP metabolites that form DNA adducts as well as deplete cellular antioxidant levels are involved in carcinogenesis (15) and lead to atherosclerosis in several experimental models. In our experiments high concentrations of BaP clearly induced necrosis without inducing apoptosis. The lack of induction of HSP70 or HO-1 by BaP is in agreement with previous studies showing that BaP does not activate HSF (42). The fact that BaP fails to induce a stress response may actually enhance its long-term toxicity, particularly its capacity to form oxidative metabolites that induce mutagenic DNA adducts and deplete intracellular antioxidant molecules, both effects that promote atherosclerosis. Furthermore, effects of BaP in the vessel wall unrelated to modulation of stress responses, such as induction of cyclooxygenase-2 (38, 45), which has antiapoptotic effects, may independently contribute to TS-mediated atherosclerosis (13).

We previously demonstrated that TS generated extracellular ROS during incubation with cells (28). To test the contribution of ROS to the induction of stress responses by TS in monocytes and endothelial cells, we treated these cells with the GSH-repleting antioxidant drug NAC before and during exposure to TS. NAC treatment protected the cells from apoptosis and decreased the stress responses induced by TS. One of the hypotheses why NAC is cytoprotective in the model of TS exposure is that NAC inhibits intracellular GSH depletion secondary to TS exposure (unpublished data), thus preventing lipid peroxidation of cellular and subcellular membranes. This hypothesis is supported by the fact that NAC and GSH have similar effects on smoking-induced oxidative alterations in phagocytes and epithelial cells (26). These results in vascular endothelial cells extend our earlier observations in that oxidative stress is involved in the TS-mediated induction of stress proteins and/or cell death in human monocytes (3). The production of ROS by monocytes leads to low-density lipoprotein oxidation and promotes lipid peroxidation, two events that are central to atherosclerosis (16). ROS present in TS could amplify the oxidative stress repeatedly applied during the process of atherogenesis. Our results further support the potential beneficial effects of antioxidants in smokers.

The authors are grateful to Dr. Françoise Russo-Marie for critical review.

The authors acknowledge financial support from Electricité de France (to M. Vayssier-Taussat), National Heart, Lung, and Blood Institute RO1 Grant HL-55627 (to B. Weksler), Institut National de la Santé et de la Recherche Médicale (to B. S. Polla), and Association Claude Bernard (to Y. Aron).

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