Enhanced protein denaturation in indomethacin-treated cells

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Abstract Indomethacin, a potent anti-inflammatory drug, activates the DNA-binding activity of human heat shock transcription factor 1 (HSF1), but this is insufficient to elevate heat shock gene expression. However, indomethacin pretreatment leads to a complete heat shock response at temperatures that are by themselves insufficient. Here, we showed that the heat-induced loss of enzymatic activity of a nuclear or a cytoplasmic luciferase expressed in murine cells was enhanced when cells had been pretreated with indomethacin. Additionally, in these cells the 70-kDa constitutive heat shock protein exhibited an enhanced aggregation in the presence of indomethacin. Similarly an increase in the aggregation of β -galactosidase was observed. These data suggest that indomethacin at moderate temperatures accelerates the presence of denatured proteins in the cell, thus lowering the temperature threshold for a heat shock response.

INTRODUCTION

The cellular response to stress conditions such as heat shock, oxidative stress, and other stresses that result in the appearance of nonnative proteins is the rapid and inducible synthesis of heat shock proteins (Hsps; reviewed in Lindquist 1986; Lindquist and Craig 1988; Voellmy 1996). Hsps, in turn, provide a stress tolerance and cytoprotection against stress-induced molecular damage (Angelidis et al 1991; Li et al 1991; reviewed in Parsell and Lindquist 1994).

In eukaryotes, expression of Hsps is regulated by the heat shock factor (HSF), which acts through heat shock elements (HSE) found in the promoters of the Hsp genes (Pelham 1982; reviewed in Morimoto et al 1996). Several HSFs have been identified in higher eukaryotes (reviewed in Wu 1995; Morimoto 1998). HSF1 and HSF3 are required for maximal heat shock responsiveness, whereas HSF2 is activated during development, differentiation, and ubiquitin-proteasome pathway inhibition (Mathew et al 1998; Tanabe et al 1998; reviewed in Morimoto 1998). In mammalian cells, HSF1 is the main heat shock-inducible factor and is subject to a complex regulation under stress conditions, namely trimerization, DNA binding, in-

ducible phosphorylation, and stress-induced transcriptional activation (Rabindran et al 1991; Baler et al 1993; Sarge et al 1993; Morimoto et al 1996).

Salicylates and other nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat inflammation and other diseases. Treatment of mammalian cells with salicylates and other NSAIDs induces formation of the HSF1 DNA binding trimer in the absence of transcriptional activity (Jurivich et al 1992, 1995). However, pretreatment with the anti-inflammatory drug indomethacin decreases the temperature threshold of the heat shock response and confers cytoprotection (Lee et al 1995). In vivo in rats, aspirin (acetyl salicylate) potentiates heat stress-induced Hsp70 expression (Fawcett et al 1997). It is unclear whether this activation of HSF1 occurs through the generation of unfolded proteins by these drugs or the mechanism is more direct, involving modification of HSF1 itself. In vitro in isolated liver microsomes, indomethacin and other NSAIDs can lead to denaturation of cytochrome P-450 into cytochrome P-420 (Falzon et al 1986).

Heat shock results in the inactivation of various cellular proteins and exogenous reporter enzymes expressed within a mammalian cell (Nguyen et al 1989; Dubois et al 1991; Pinto et al 1991; reviewed in Bensaude et al 1996). This inactivation has been found to be due to their insol-

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Fig 1. Effect of indomethacin on heat inactivation of cytoplasmic and nuclear luciferase. (A) Cytoplasmic luciferase. (B) Nuclear luciferase. RL/V3 (Fig 1A) and RNLSL/V6 (Fig 1B) clonal cell lines were isolated after G418 selection of NIH 3T3 cells cotransfected with either pRSVLL/V or pRSVnlsLL/V and pSVtkneo, respectively. Plasmid pRSVnlsLL/V was described in Michels et al (1995), and pRSVLL/V was provided by S. Subramani (Gould et al 1989). These clonal cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, treated with trypsin, and distributed equally into cell culture tubes. The following day, cells were treated with 0 mM (open square), 0.25 mM (closed square), 0.5 mM (closed circle), and 1 mM indomethacin (closed triangle) for 30 minutes at 37°C and then heated at 41°C for various times in the same medium. Indomethacin (Sigma, Germany) was dissolved in dimethyl sulfoxide to a concentration of 50 mM just before addition to the cells and added to the culture medium containing 20 mM morpholinopropanesulfonic acid, pH 6. As a control, the same concentration of dimethyl sulfoxide was added. Before the heat shock, the tubes were preincubated in a water bath at 37°C and transferred to a second water bath at the indicated temperatures. After the heat shock, the cells were chilled and washed with ice-cold phosphate-buffered saline and lysed in a tube using 0.5 mL lysis buffer A (25 mM Tris/PO₄, pH 7.8, 10 mM MgCl₂, 1% Triton X-100, 1 mM ethylenediamine-tetraacetic acid, 0.05% 2-mercaptoethanol, 15% glyc-

ubilization. Firefly luciferase, bacterial β -galactosidase, the 70-kDa constitutive Hsp, and the 68-kDa dsRNA-dependent protein kinase are found mostly in the supernatant fraction of centrifuged lysates from control unshocked mammalian cells. However, when cells are lysed after heat shock, a proportion of the reporter molecules is found to be aggregated to the nuclear pellets (Dubois et al 1991; Pinto et al 1991). This insolubilization does not affect all cellular proteins; many of them remain unaffected by heat shock. The heat-induced loss of solubility and enzymatic activity were taken as indicators of protein denaturation; both are reversible and attenuated in thermotolerant cells (Nguyen et al 1989; Pinto et al 1991).

The aim of this work is to examine whether indomethacin, as a representative of NSAIDs, influences protein denaturation in the cell. Transfected exogenous proteins as well as a cellular protein were used as reporters. Indomethacin was found to enhance heat insolubilization of these reporter proteins.

RESULTS

Cytoplasmic and nuclear luciferase

Firefly luciferase is inactivated on mild heat shock when it is expressed in mammalian cells. Such loss of enzyme activity is accompanied by loss of solubility, which is taken as an indication of protein denaturation (Nguyen et al 1989; Pinto et al 1991). Murine cells stably transfected with pRSVLL/V (Gould et al 1989), which express cytoplasmic luciferase, were investigated first. At 37°C, the presence of the drug had no effect (data not shown). At 41°C, in control cells (0 mM indomethacin) luciferase activity declined with a rate that was increased about 3-fold on exposure to 0.5 mM indomethacin. In the presence of 1 mM indomethacin, a remarkable increase of about 8-fold in the rate of inactivation was observed (Fig 1A).

We next used luciferase targeted to the nucleus to investigate whether indomethacin treatment could interfere with the nuclear environment. Nuclear luciferase was more rapidly heat inactivated than was cytoplasmic luciferase, as described before (Michels et al 1995). However, both cytoplasmic and nuclear luciferases were heat inactivated at similar rates in vitro (Michels et al 1995). At 41°C, luciferase activity declined with a rate that was increased 2-fold on exposure to 0.25 mM indomethacin and about 3-fold on exposure to 0.5 mM indomethacin.

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erol. Next, 0.15 mL cell lysate was mixed with 0.1 mL buffer A containing 1.25 mM adenosine triphosphate and 93 mg/mL luciferin (Sigma) just prior to measurement. Luciferase activity in lysates was measured in a luminometer (Interbio, The Woodlands, TX, USA) and was plotted as a percentage of the activity before heat shock (100%).

Fig 2. Indomethacin enhances the heat-induced insolubilization of β -galactosidase (Western blot test). (A) Pellet fractions. (B) Supernatant fractions. G2b2 clonal cell line was grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. This cell line was isolated after G418 selection of NIH 3T3 cells cotransfected with the plasmids pCMV β GAL and pSVtkneo. Plasmid pCMV β GAL was elaborated by inserting the *HindIII/BamHI*, β -GAL-containing fragment of pCH110 into the *HindIII/BamHI* sites of pCMVLuc. Individual 60-mm plates of logarithmically growing G2b2 cells were treated with 0 mM indomethacin (Fig 2A, lanes 1, 4, 7, 10, and Fig 2B, lanes 1, 4, 7, 10), 0.5 mM indomethacin (Fig 2A, lanes 2, 5, 8, 11, and Fig 2B, lanes 2, 5, 8, 11), and 1 mM indomethacin (Fig 2A, lanes 3, 6, 9, 12, and Fig 2B, lanes 3, 6, 9, 12) for 30 minutes at 37°C, then were sealed and immersed in a water bath at the indicated temperatures for 30 minutes before the cell lysis. Indomethacin was used as in Figure 1. After the treatment dishes were rinsed with ice-cold phosphate-buffered saline, cells were scraped off dishes in a small volume of phosphate-buffered saline, transferred to Eppendorf tubes, collected by centrifugation, and lysed in a buffer B (1% Triton X-100, 10 mM MgCl₂, 50 mM Tris-Cl, 50 mM NaCl, 0.5% 2-mercaptoethanol). The lysates were fractionated by centrifugation at 12 000 $\times g$ at 4°C for 10 minutes into supernatants (Fig 2B) and pellets (Fig 2A). The pellets were washed 3 times with buffer B, dissolved in 3 $\times$ Laemmli sample buffer, heated for 5 minutes at 100°C, and electrophoresed on a 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Laemmli sample buffer was added to the supernatant fractions. Samples corresponding to the same number of cells were analyzed on 8% SDS-PAGE. The gels were electrotransferred onto nitrocellulose (Amersham, Buckinghamshire, UK) and hybridized to a mouse monoclonal anti- β -galactosidase antibody (Promega, Madison, WI, USA). The blot was incubated with the anti-mouse secondary antibody (horseradish peroxidase conjugated; Amersham) and developed with Supersignal substrate Western blotting (Pierce, Rockford, IL, USA).

In the presence of 1 mM indomethacin, an increase of about 4-fold in the rate of inactivation was observed (Fig 1B).

In conclusion, the inactivation rate of both cytoplasmic and nuclear luciferases was increased in the presence of indomethacin during heat shock.

β -Galactosidase

Another reporter protein, β -galactosidase, was next investigated. Murine cells stably transfected with bacterial β -galactosidase were subjected to indomethacin treatment. β -Galactosidase from *Escherichia coli* is partially insolubilized during heat shock when expressed in murine

cells (Nguyen et al 1989; Pinto et al 1991). The amount of β -galactosidase in the soluble fractions was almost unchanged in all conditions used (Fig 2B, lanes 1–12). In control cells (0 mM indomethacin) under non-heat shocked conditions, most of the β -galactosidase was found in the soluble fraction (Fig 2B, lane 1) and very little in the insoluble (Fig 2A, lane 1). When cells were incubated at 41°C in the absence of the drug, the amount of β -galactosidase in the pellet did not change, whereas in the presence of 0.5 mM or 1 mM indomethacin, a large amount of β -galactosidase was insolubilized (Fig 2A, lanes 4–6). In the presence of 0.5 mM indomethacin at 41°C, a 2-fold increase in the pellet fraction was observed, while in the presence of 1 mM indomethacin at 41°C, a

Table 1 Quantification of the β -galactosidase and hsc70 molecule distribution in the insoluble fractions of indomethacin-treated cells

		Temperature (°C)											
		37			41			42.5			44		
Indomethacin		0	0.5	1.0	0	0.5	1.0	0	0.5	1.0	0	0.5	1.0
			mM			mM			mM			mM	
β -galactosidase	1	1	1.3	1.2	1.3	3	6	11	16	16	34	44	36
hsc70	1	1	1.5	1.6	2.8	8	16	10	16	22	28	45	48

Data shown result from the scanning of the autoradiographs presented in Figure 2A and Figure 3A. Densities in bands of the insoluble fractions were measured in a densitometry scanner (Sebia). The band density in control cells (0 mM indomethacin) at 37°C was taken as 1.

4-fold increase was observed (Fig 2A, lanes 4–6; Table 1). At 42.5°C in the absence of the drug, a major increase in the insoluble fraction was observed and was slightly enhanced in the presence of 0.5 mM or 1 mM indomethacin (Fig 2A, lanes 7–9; Table 1). At 44°C in the absence of the drug, a large amount of β -galactosidase was insolubilized, which was not significantly increased in the presence of indomethacin (Fig 2A, lanes 10–12; Table 1).

Hence, heat insolubilization of the reporter protein β -galactosidase was also enhanced in indomethacin-treated cells.

Hsc70

We then asked whether the enhanced insolubilization of proteins such as luciferase and β -galactosidase also included cellular proteins.

Hsc70 is a member of the 70-kDa Hsp family, which is constitutively expressed and abundant in the unstressed cell. It is one of the major insolubilized proteins in heat-shocked mammalian fibroblasts (Dubois et al 1991). We investigated the Hsc70 cellular distribution under the conditions of indomethacin treatment used above. In lysates of control cells (0 mM indomethacin), most of the protein was found in the soluble fraction (Fig 3B, lane 1) and very little in the insoluble fraction (Fig 3A, lane 1). The protein levels in the soluble fractions were almost unaffected by the heat treatment (Fig 3B, lanes 1–12). However, at 41°C with 0.5 mM indomethacin treatment, a 3-fold increase in the amount of protein found in the pellet fraction of cells was observed. This increase was more profound, about 5-fold, when cells were treated with 1 mM indomethacin (Fig 3A, lanes 4–6; Table 1). At 42.5°C, there was only a 2-fold increase in the amount of insolubilized protein with 1 mM indomethacin (Fig 3A, lanes 7–9; Table 1). At 44°C, there was no significant difference (Fig 3A; lanes 10–12; Table 1).

Thus, the enhancement in the heat induced insolubilization that was observed in reporter proteins was seen with at least 1 cellular protein, namely Hsc70.

DISCUSSION

Indomethacin, as with other NSAIDs, alters the expression of the human heat shock genes. In particular, indomethacin reduces the temperature threshold of the heat shock response.

In the current study, we examined the effect of indomethacin in the heat-induced denaturation of proteins. We observed an enhancement of heat insolubilization in β -galactosidase and Hsc70 and inactivation of luciferase expressed either in the cytoplasm or in the nucleus. Although this enhanced protein denaturation was observed at mild heat shock temperatures, the presence of indomethacin at 37°C had no effect on protein denaturation. These observations suggest that indomethacin acts differently under mild heat stress than at physiological conditions (37°C). This may have direct implication for inflammation, which is accompanied by local rises in temperature.

It is generally accepted that all inducers of heat shock genes share a common mechanism, namely abnormal proteins (Ananthan et al 1986). Our data suggest that indomethacin accelerates the generation of denatured proteins under temperatures moderately above normal. This can be involved in the therapeutic action of indomethacin, although the concentrations used therapeutically are below the ones we used (Vane and Botting 1987). Alternatively, it can be implicated in its toxicity. It would be interesting to examine whether salicylates or other NSAIDs affect protein denaturation as well. It is possible that all NSAIDs function similarly at least in relation to inflammation. However, it is noteworthy that aspirin inhibits prostaglandin H synthase, whereas salicylic acid does not (Siegel et al 1979). Furthermore, salicylates and aspirin but not indomethacin inhibit the activation of transcription factor NF- κ B in human cells (Kopp and Ghosh 1994).

Sodium salicylate decreases the intracellular adenosine triphosphate (ATP) levels in *Drosophila* tissue culture cells and induces HSF binding in a dose-dependent manner (Winegarden et al 1996). On the other hand, in ATP-depleted mammalian cells an increased thermal aggregation, has been observed with 4 different reporter proteins

Fig 3. Indomethacin enhances the heat-induced insolubilization of hsc70 (Western blot). (A) Pellet fractions. (B) Supernatant fractions. NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Individual 60-mm plates of logarithmically growing cells were treated with 0 mM indomethacin (Fig 3A, lanes 1, 4, 7, 10, and Fig 3B, lanes 1, 4, 7, 10), 0.5 mM indomethacin (Fig 3A, lanes 2, 5, 8, 11, and Fig 3B, lanes 2, 5, 8, 11) and 1 mM indomethacin (Fig 3A; lanes 3, 6, 9, 12, and Fig 3B, lanes 3, 6, 9, 12) as in Figure 2. After the treatment, cells were lysed and fractionated into supernatants and pellets as in Figure 2. Supernatant and pellet fractions were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gels were electrotransferred onto nitrocellulose (Amersham, Buckinghamshire, UK) and hybridized to the anti-hsc70 affinity purified rat polyclonal antibody (SPA-815, Stressgen, Victoria, BC, Canada). The blot was incubated with anti-rat secondary antibody (horseradish peroxidase conjugated; Amersham) and developed as in Figure 2.

as well as with Hsc70 (Nguyen and Bensaude 1994). Indomethacin could decrease the intracellular ATP levels in mammalian cells, thus enhancing protein denaturation of reporter proteins. However, there is no evidence so far that NSAIDs interfere with the intracellular ATP levels in mammalian cells.

These results suggest that indomethacin at moderate temperatures favors protein denaturation in the cell. This could be a possible explanation for the mode of action of indomethacin and perhaps of other anti-inflammatory drugs in lowering the temperature threshold for a heat shock response.

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