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RAT CONTRAPSINS ARE THE TYPE II ACUTE PHASE PROTEINS: REGULATION BY INTERLEUKIN 6 ON THE mRNA LEVEL

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Three highly homologous serine protease inhibitors, SPI-1, SPI-2 and SPI-3 (contrapsins), are synthesized in rat liver. Their expression is regulated differently in healthy and inflamed animals. We found that interleukin 6 (IL-6), a major acute phase cytokine, and to a lesser extent leukemia inhibitory factor (LIF), both together with glucocorticoids, are responsible for the regulation of expression of the contrapsins in rat hepatocytes in primary culture. The effect of IL-6 is time- and dose- dependent. IL-1, TGFβ_1, HGF, PMA and IL-8 did not have any effect on contrapsin mRNA levels. We postulate that SPI-1, SPI-2 and SPI-3 belong to the class II acute phase proteins. Additionally, we show induction of SPI-3 mRNA in rat liver by *in situ* hybridization using a specific oligonucleotide probe. © 1994 Academic Press, Inc.

Mammalian plasma contains a number of protease inhibitors controlling a variety of physiological processes such as complement activation, blood coagulation and some aspects of the inflammatory response [7]. Among plasma inhibitors, serpins (serine protease inhibitors) belong to a group of proteins controlling the action of serine proteases [23]. The serpin superfamily includes amongst others α_1-proteinase inhibitor (α_1PI), α_1-antichymotrypsin (ACT), antitrombin III, and C1 inhibitor. Leukocyte elastase is the target enzyme for one of the most abundant plasma proteinase inhibitors α_1PI, which in man also inhibits trypsin. Mouse α_1PI however inhibits chymotrypsin, elastase and thrombin but has no effect on trypsin [20, 21, 22]. New inhibitors of trypsin called contrapsins were purified from mouse, rat and guinea pig plasmas and their inhibitory spectra were characterized [5, 19, 22]. Three contrapsins SPI-1, SPI-2 and SPI-3 were cloned from a rat liver cDNA library and their nucleotide sequences were determined

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Abbreviations: FCS, Fetal Calf Serum; HGF, Hepatocyte Growth Factor; LPS, Lipopolisaccharide; PMA, phorbol 12-myristate 13-acetate; TGF, Tumor Growth Factor.

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[5, 10, 11]. The genes, including the promoter regions, of rat contrapsins have been recently cloned [12] and proteins binding to the promoter regions have been partially characterized [15].

Two contrapsins, SPI-1 and SPI-2, are expressed in the liver of healthy rat. Their expression is controlled by growth hormone [25] and glucocorticoids [6, 13] and decreases under inflammatory conditions [6, 8, 10]. SPI-3 mRNA is hardly detectable in the liver of healthy animals, but is rapidly induced during acute inflammation caused by turpentine injection [10]. It was suggested that inflammatory cytokines are responsible for this differential regulation of contrapsin expression. In preliminary experiments the effect of hepatocyte stimulating factors (HSF) released from human squamous carcinoma cell line (COLO-16) was demonstrated [1]. Possible IL-6 - responsive elements were identified in the promoter region of SPI-3 gene [15], but clear evidence has yet to be provided showing which cytokines are involved in the regulation of contrapsin expression. Furthermore, no difference was found in the binding of transcription factors present in the nuclear extracts prepared from the livers of healthy and inflamed rats [15]. We show in this report that SPI-1, SPI-2 and SPI-3 belong to the class II acute phase proteins, the expression of which is controlled by IL-6 (and LIF) together with glucocorticoids. IL-1 has no effect on contrapsin expression. Moreover, we demonstrate regulation of contrapsin expression in the liver by *in situ* hybridization.

Materials and Methods

Materials: Restriction enzymes, poly(A) polymerase and the random primed DNA labeling kit were purchased from Boehringer-Mannheim (Mannheim, Germany). [α-^{32}P]dCTP (110 TBq/mmol) was obtained from Amersham International (Amersham, UK). [α-^{35}S]dATP (37 -55.5 TBq/mmol) was from NEN (NEN, Germany). *Escherichia coli LPS* and PMA were from Sigma (Munich, Germany). rhIL-6 was obtained from Dr. Stefan Rose-John (Aachen, Germany), rhLIF was from Dr. Heinz Baumann (Buffalo, NY), rhIL-1α was from Genzyme (Cambridge, MA), murine recombinant IL-8 was from PropoTech Inc. (Rocky Hill, NY), rhHGF was from Dr. Toshikazu Nakamura (Osaka, Japan) and rhTGFβ_1 was from Dr. Carl-Henrik Heldin (Uppsala, Sweden). The plasmid containing mouse contrapsin cDNA [18] was kindly provided by Dr. Hyogo Sinohara (Osaka, Japan). Full length cDNA was used for random priming. The following oligonucleotides specifically recognizing SPI-1, SPI-2 and SPI-3 were synthesized:

5'-GGTTGAAATTCAGAGTTCGAGGTTGTCTAC-3' (SPI-1).
5'-GAAGAGGTATAGTTTGCGGTAAACTTTTTA-3' (SPI-2),
5'-AGCTATAATCAGAGGGTCCAGTTTTGCAGA-3' (SPI-3),

Animals and cell culture: Male Wistar rats of 200-300 g body weight were injected intraperitoneally with 10 mg LPS per kg body weight. 2 and 24 hours after LPS injection, animals were sacrificed, livers immediately removed and used for RNA isolation or preparing sections for *in situ* hybridization. Primary rat hepatocytes were prepared by perfusion of the liver [2]. Hepatocytes were cultured in Williams E medium supplemented with 5% FCS, antibiotics, 1 μM insulin and dexamethasone (10^{-7} M). Cells were stimulated 48 hours after starting the culture.

RNA preparation and Northern blot analysis: Total RNA was prepared using the phenol extraction method [14, 17]. 5 μg of RNA were heated to 65°C for 10 min. in 50% formamide, 20 mM morpholinopropanesulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde prior to gel electrophoresis in 1% agarose containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde. Equal loading of the RNA gel was checked by ethidium bromide staining of 18S and 28S ribosomal RNA. The separated RNA was transferred

to Hybond-N membranes (Amersham, UK) according to manufacturer's instructions. The filters were prehybridized at 68°C for 3 h in 10% dextran sulphate, 1 M sodium chloride, 1% SDS and hybridized in the same solution with the cDNA fragment labeled by random priming [3]. After hybridization, non-specifically bound radioactivity was removed by washing in 2x standard saline solution (SSC) at room temperature, followed by two subsequent washes in 2xSSC/1% SDS at 68°C for 30 min each. The filters were then subjected to autoradiography using intensifying screens.

In situ hybridization: Livers of rats were rapidly removed and frozen on dry ice. Ten consecutive series of sections (5 μm thick) were made on a cryostat (Shandon, UK) and thaw-mounted onto chromealum pretreated slides, postfixed in 4% paraformaldehyde for 10 min. and processed for an *in situ* hybridization as described [26]. Sections were hybridized in 50% formamide, 4xSSC, 10% dextran sulphate, 0.02% Ficoll 400, 0.02% BSA, 0.02% polivinylpirrolidine containing 1 mg/ml salmon sperm DNA and 0.1 mg/ml yeast tRNA. Synthetic oligonucleotides were labeled with [α-^{35}S]dATP using poly(A) polymerase [16]. To visualize silver grains over individual cell bodies, the slices were immersed in a Kodak NTB-3 autoradiographic emulsion, left for 30 days at 4°C and then developed in D-19 developer and fixed.

Results

Adult male Wistar rats were injected with LPS and total RNA was prepared from the livers after 2 or 24 hours. The abundance of contrapsin mRNA was determined by Northern blot analysis. Fig. 1 shows transcripts of approx. 1.8, 2.2 and 5 kb in size. The 2.2 and 5 kb transcripts were hardly detectable in the liver of healthy animals but were induced already 2 hours after administration of LPS. Full induction of the 2.2 transcript was achieved after 24 hours. The fast migrating transcript of 1.8 kb hybridizing with the mouse probe was constitutively expressed in the liver of healthy animals and induced 2 hours after injection of LPS but completely disappeared 22 hours later.

In order to elucidate the regulation of contrapsin expression in the liver, we prepared primary rat hepatocyte cultures and stimulated the cells with factors known to affect gene expression in liver during inflammation. The measurement of contrapsin expression by Northern blot analysis revealed the presence of 2.2 and 1.8 kb transcripts in control hepatocytes but the smaller transcript was barely detectable (Fig. 1). Moreover, the relative amounts of both transcripts were slightly different in particular experiments (Fig. 2). We found that IL-6, the major regulator of the expression of acute phase proteins, was responsible for the dramatic stimulation of the 2.2 and 5 kb transcripts. LIF was also able to stimulate both transcripts but to a lesser extent. Other stimulators: IL-1, HGF, TGFβ_1, PMA and IL-8 were ineffective. The fast migrating transcript (1.8 kb) was also induced by IL-6, which could be easily seen after a long exposure of the filter (data not shown).

The dose- and time-dependence of contrapsin mRNA induction by IL-6 is shown in Fig. 2. The 2.2 and 5 kb transcripts were fully induced 24 hours after incubation with IL-6 and the amount of 1.8 kb transcript increased with similar doses. However, the time dependence shows the induction of this transcript only during the first 24 hours. After an additional 24 hours, the amount of the 1.8 kb transcript was comparable to that observed in the control cells (Fig. 2A). Eighteen hours after stimulation with IL-6, a slight induction of the 2.2 and 5 kb transcripts was already observed

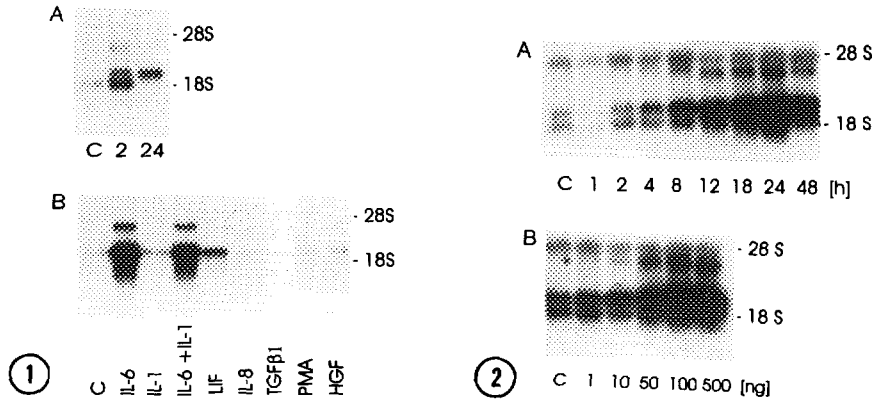

Fig. 1. Regulation of contrapsins mRNAs in the rat liver and primary hepatocytes. (A) Rats were injected intraperitoneally with 10 mg LPS per kg body weight. After 2 and 24 hours RNA was prepared from rat livers and subjected to Northern blot analysis using a cDNA probe coding for murine contrapsin. (B) Primary rat hepatocytes were prepared as described in Materials and Methods and stimulated with IL-6 (50 ng/ml), IL-1 (100 u/ml), LIF (10 u/ml), IL-8 (5 ng/ml), $TGF\beta_1$ (10 ng/ml), PMA (100 ng/ml) or HGF (10 ng/ml) for 18 hours. Isolated RNA was analyzed by Northern blotting.

Fig. 2. Time- and dose-dependence of contrapsin mRNAs induction by IL-6. Primary rat hepatocytes were stimulated with 50 ng/ml of human recombinant IL-6 for the times indicated (A) or for 18 hours with an increasing amount of IL-6 (B). RNA was isolated and used for Northern blot analysis.

at a concentration of 1 ng/ml, whereas maximal 2.2 and 5 kb mRNA levels were achieved at 100 ng/ml of IL-6 (Fig. 2B).

To understand the regulation of the three rat contrapsins reported so far (hybridizing with a mouse probe) we synthesized three specific oligonucleotide probes. The probes were used in an *in situ* hybridization analysis with the livers of control and inflamed rats. An *in situ* hybridization histochemistry study revealed that all three contrapsin genes examined appear to be expressed in rat liver (data not shown). Fig. 3 shows that the SPI-3 mRNA is uniformly distributed in the hepatocytes of control rats and is already induced 2h after administration of LPS, and profoundly elevated when measured after 24 hours. However, we were not able to show any significant change in the amount of SPI-1 and SPI-2 mRNAs by this method.

Discussion

As reported previously, contrapsin expression is differently regulated during acute inflammation caused by turpentine injection [1, 6, 8]. It was established, by using specific oligonucleotide probes, that SPI-3 is encoded by a 2.2 kb transcript while SPI-1 and SPI-2 are both encoded by two different transcripts of the same size (1.8 kb) [10]. We analyzed rat contrapsin mRNA levels (hybridizing with a mouse probe) 24 hours (reported earlier) and a short time (2 hours) after LPS injection. We found that rapid induction of three transcripts (5, 2.2 and 1.8 kb) had already occurred 2 hours after LPS treatment. The 1.8 transcript was hardly detectable 24 hours after LPS injection which is in a good agreement with previous data .

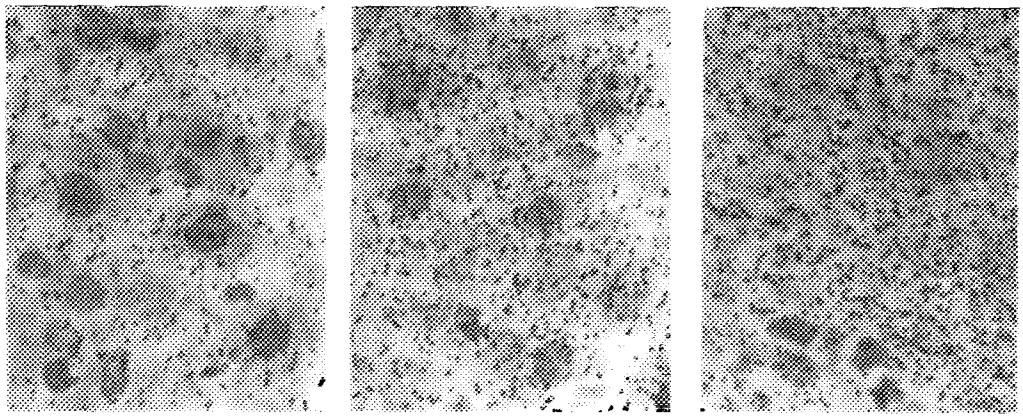

Fig. 3. Localization of SPI-3 mRNA in rat liver. Rats were injected intraperitoneally with 10 mg LPS per kg body weight. After 2 and 24 hours livers were removed, prepared for *in situ* hybridization as described in Materials and Methods and probed with a SPI-3 specific oligonucleotide.

In order to determine which of the known factors are responsible for the regulation of SPI genes, we performed *in vitro* experiments with primary rat hepatocytes in culture. We noticed that control cells expressed the 2.2 kb transcript and lesser amounts of 1.8 kb transcript, due to the fact that the cells were cultured in medium containing dexamethasone which is known to regulate the expression of SPI genes [6, 13]. The same effect was earlier described for rat hepatocytes in primary culture [1]. We used different stimulators of acute phase proteins and only IL-6 (and LIF) regulated SPI expression while IL-1, alone or together with IL-6 had no effect. All this evidence suggests that rat contrapsins belong to the class II acute phase proteins regulated by IL-6 (and LIF) and glucocorticoids. In all our experiments we observed parallel coexpression of the 5 and 2.2 kb transcripts. The same phenomenon can be seen when analyzing the expression of 28 and 20S mRNA encoding contrapsins in different mouse strains [4], and it is likely that the 5 kb transcript is a not spliced form of the 2.2 kb transcript. It is significant that the amount of SPI-3 mRNA (2.2 kb transcript) increased dramatically after IL-6 treatment. In rat, other inhibitors of proteinases such as α_2-macroglobulin, cysteine protease inhibitor (T-kininogen) and the tissue inhibitor of metalloproteinases are upregulated by IL-6 as well. The fact that three highly homologous contrapsins characterized so far are regulated so differently, is probably due to their inhibitory spectra resulting from different reactive sites.

In situ hybridization showed that cells producing SPI-1, SPI-2 and SPI-3 are uniformly distributed in liver. The response of SPI-3 measured *in situ*, using a specific oligonucleotide probe, was in agreement with the data obtained from Northern blot analysis using a mouse cDNA probe and a specific oligonucleotide probe (data not shown).

Further characterization of the IL-6 dependent response of SPI genes needs to be worked out in future. Due to the fact that SPI mRNAs are already regulated 2 hours after the administration of LPS and their expression is regulated by IL-6 and LIF, it is probable that the recently

characterized acute phase response factor (APRF) [27] and Jak-Tyk protein kinases [9] are involved in the regulation of SPI genes.

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