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Inverse α_{1A} and α_{1D} adrenoceptor mRNA expression during isolation of hepatocytes

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Abstract

It is now well documented that changes in gene expression take place during cell isolation and culture. Here, we report the change in the expression of the mRNAs for α_1-adrenoceptor subtypes, during dissociation of guinea pig liver cells with collagenase.

Using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assays, it was observed that during the isolation procedure, the mRNA for the α_{1A}-adrenoceptor, normally expressed in whole liver, was degraded and the mRNA for α_{1D} subtype, barely expressed in whole liver, increased in an actinomycin D-sensitive manner. When the isolation procedure was performed in the presence of cycloheximide, the mRNA for the α_{1A}-adrenoceptor did not diminish and the induction of the α_{1D}-adrenoceptor mRNA was even more evident. Our data indicate that cell isolation alters α_1-adrenoceptor mRNA expression.

Keywords: α_1-Adrenoceptor; Hepatocyte; Liver gene expression

1. Introduction

The liver constitutes a unique, multicellular organ whose functions are crucial for the overall physiology of the organism. The natural catecholamines (adrenaline and noradrenaline) regulate a wide variety of such functions including modulation of key steps in carbohydrate, lipid, and amino acid metabolism. In the adult rat liver, the best studied hepatic model, adrenoceptors of the α_1 and β_2 subtypes are present, but functionally α_1-adrenoceptors mediate the adrenergic effects almost exclusively (Exton, 1980).

α_1-Adrenoceptors constitute a heterogeneous subfamily of G protein-coupled receptors that on the basis of cloning and pharmacological data, can be subdivided into three major subtypes: α_{1A}, α_{1B}, and α_{1D} (Hieble et al., 1995). It has been shown that the genes for these adrenoceptors are expressed in discrete, tissue-specific patterns in the same organism (see for example Price et al., 1994), but it is also becoming clear that the same tissue may express distinct α_1-adrenoceptor subtypes in different species. Thus, it has been observed that the livers of rabbits (García-Sáinz et al., 1992a), dogs (García-Sáinz et al., 1995b), cats (García-Sáinz et al., 1996a) and humans (García-Sáinz et al., 1995c) mainly express α_{1A}-adrenoceptors whereas expression of the α_{1B} subtype has been detected in the livers of a fish (*Ichtalurus punctatus*) (García-Sáinz et al., 1995a), the domestic fowl (Gutiérrez-Venegas and García-Sáinz, 1993), and rodents such as rats, mice, and hamsters (García-Sáinz et al. 1994). Coexpression of α_{1A}- and α_{1B}-adrenoceptors has been observed in the liver of monkeys (García-Sáinz et al., 1996b).

The α_1-adrenoceptor subtype expressed in the guinea pig liver has been especially difficult to characterize. Pharmacological and radioligand binding analysis indicated the presence of α_{1A}-adrenoceptors in hepatocytes (García-Sáinz et al., 1992a,b, 1995d). However, when Northern analysis was performed with RNA from guinea pig hepatocytes, there was hybridization signal with the α_{1D} but not with the α_{1A}-adrenoceptor probe (García-Sáinz et al., 1992a). We have observed that isolation of metabolically active guinea pig hepatocytes by proteolytic digestion of the extracellular matrix of the liver, results in immediate degradation of α_{1A} mRNA and in an increased transcription of the α_{1D}-adrenoceptor gene. To our knowledge, this constitutes the first example of transition of α_1-adrenoceptor subtype mRNA expression in any hepatic model.

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2. Materials and methods

Actinomycin D, cycloheximide, trypan blue, ethidium bromide, guanidinium thiocyanate, *N*-lauroylsarcosine, phenol, isoamyl alcohol, chloroform, sodium dodecyl sulfate, EDTA, β-mercaptoethanol, polyvinylpyrrolidone and the reagents used for polyacrylamide gel electrophoresis were obtained from Sigma. Nylon membranes (Hybond) were from Amersham. Restriction enzymes were from New England Biolabs. Reverse Transcription-Polymerase Chain Reactions (RT-PCR) were performed using Perkin Elmer Kits. Nick translation kits were from Gibco BRL. [α-^{32}P] dCTP (3000 Ci/mmol) was from DuPont New England Nuclear.

Male guinea pigs (*Cavia porcellus*) of 250–300 g were fed ad libitum and maintained and sacrificed according to the principles and guidelines of our Institute, Health Department (Mexico) and the NIH, to avoid unnecessary pain to the animals. To obtain samples of total liver, animals were sacrificed and a sample of 1g of hepatic tissue was taken. Hepatocytes were isolated by perfusing the liver with collagenase followed by mechanical disruption (Berry and Friend, 1969). The cells were incubated in Krebs–Ringer bicarbonate buffer at 37°C under an atmosphere of 95% O_2/5% CO_2, pH 7.4 in an orbital water bath shaker during 15 or 30 min after isolation. Cell viability was routinely $>$ 90% as evidenced by trypan blue exclusion. Samples (of fresh liver, perfused liver, liver digested with collagenase for 10 min, recently isolated liver cells and hepatocytes incubated for 15 or 30 min) were taken to obtain RNA.

Where indicated, animals were treated with actinomycin D (30 μg/animal, i.p.) or cycloheximide (500 μg/animal, i.p.) 1 h before the isolation of the liver. Liver was perfused and hepatocytes were isolated in buffers containing actinomycin D (1 μg/ml) or cycloheximide (50 μg/ml). These treatments did not affect the viability of the isolated cells, as evidenced by trypan blue exclusion. These doses and concentrations have been shown to effectively inhibit transcription and protein synthesis (Ettienne et al., 1988).

Total RNA was isolated as described by Chirgwin et al (1979) with the modifications made by Le Huërou et al. (1990) and repurified (Chomczynski and Sacchi, 1987). The use of these procedures was important to preserve the integrity of RNA (routinely checked by electrophoresis on formaldehyde-containing agarose gels and visualization under UV light using ethidium bromide staining).

Total RNA (50 μg per lane) was subjected to electrophoresis on agarose-formaldehyde gels and transferred to Hybond nylon membranes. Filters were hybridized under high stringency conditions (Heindenreich et al., 1993) with 1 μg of the respective probe labeled by nick translation (specific activity = 10^8 cpm/μg). The specific probes used were: the 1.28 kb *Nco*I–*Hin*dIII fragment of the bovine α_{1A} cDNA (Schwinn et al., 1990) the complete cDNA of the rat α_{1D} subtype (Lomasney et al., 1991) and the complete cDNA of the rat GAPDH (Fort et al., 1985). Filters were exposed to Kodak Biomax™ films.

Total RNA (5 μg) was subjected to reverse transcription-polymerase chain reaction (RT-PCR). The reverse-transcription step was performed for 1 h at 42°C. Polymerase chain reaction conditions were as follows: a hot start step of 5 min at 95°C followed by 1 min at 94°C, 1.5 min at 55°C, 1.5 min at 72°C during 35 cycles and a final elongation step of 7 min at 72°C, using the following primers: 5′ TCTGGGCGGCGGTGGACGTCCTGTGCT 3′ (coding sense) and 5′ ACGTAGACCCGGCAGTACATGACCAGGATG 3′ (anticoding sense) corresponding to bases 302–329 and 597–627 of the bovine α_{1A}-adrenoceptor cDNA (Lomasney et al., 1991); 5′ CAACCGCCACCTACAGACGGTCACCAACTA 3′ (coding sense) and 5′ GCTGCGTGCGACCACGTACACGCGGCAGTA 3′ (anticoding sense) corresponding to bases 355–385 and 807–836 of the rat α_{1D}-adrenoceptor (Schwinn et al., 1990); 5′ TCCCTCAAGATTGTCAGCAA 3′ (coding sense) and 5′ AGATCCACAACGGATACATT 3′ (anticoding sense), for amplification of a 309 bp fragment of the rat GAPDH gene (Fort et al., 1985). In order to confirm the identity of the RT-PCR products, they were separated in a 7.5% acrylamide gel, denatured, neutralized and transferred onto nylon membranes; the filters were hybridized and washed under the same high stringency conditions and the probes described above. They were exposed to Kodak Biomax™ films in order to obtain an autoradiographic image.

The RT-PCR products putatively corresponding to the guinea-pig α_{1A}- (325 bp) and α_{1D}-adrenoceptors (481 bp) were cloned on the SrfI site of the plasmid, pCRSK (+), using T4 DNA polymerase and Stratagene cloning kits. Positive clones were sequenced using an AMBIOS System. The sequences obtained were analyzed using the different options of the PC/Gene™ program (IntelliGenetics, release 6.60, 1991). Sequences were aligned using the on-line service of the G-protein coupled receptor database (Kolakowski, 1994) using FASTA and BLASTN programs.

The respective RT-PCR reactions were done in the presence of 1 μCi of [α-^{32}P] dCTP. A fraction of the product (usually 5% of total reaction volume for α_1-adrenoceptors and 2% for GAPDH) was separated on 1.5% agarose gels, transferred onto nylon membranes and exposed to a Phosphor-Screen™ (Molecular Dynamics). The data were analyzed with a STORM 40™ System. After their analysis filters were exposed to X-Omat film (Kodak) and processed in order to obtain an autoradiographic image. Densitometric data were normalized to the GAPDH signal and they are presented as the mean ± S.E.M.

3. Results

Total RNA isolated from whole guinea pig liver was subjected to electrophoresis in formaldehyde-agarose gels

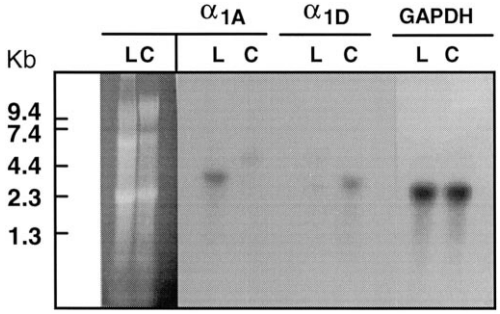

Fig. 1. Northern analysis of total RNA isolated from whole liver (L) or isolated liver cells (C) using α_{1A}-adrenoceptor, α_{1D}-adrenoceptor or GAPDH-specific probes. Total RNA was isolated as described and 25 μg were separated in a formaldehyde-containing agarose gel and stained using ethidium bromide (left panel). RNA was transferred onto nylon membranes and hybridized under high stringency conditions against α_1-adrenoceptor subtype-specific or GAPDH probes labeled by nick translation. In the right panel, a representative autoradiography is presented (5 days exposure to BIOMAX™ film).

(Fig. 1, left panel) and hybridized under high stringency conditions against specific α_{1A}-adrenoceptor, α_{1D}-adrenoceptor or GAPDH probes. A clear positive signal for the α_{1A}-adrenoceptor was observed but no signal for the α_{1D}-adrenoceptor was detected. In contrast, when Northern analysis was done with total RNA obtained from isolated hepatocytes, the hybridization signal was positive for the α_{1D}-adrenoceptor but not for the α_{1A}-adrenoceptor (Fig. 1, right panel). These data strongly suggested that a change in the expression of α_1-adrenoceptors could be taking place during hepatocyte isolation. No change was observed in GAPDH expression between liver and isolated cells.

With the aim to further document this finding RT-PCR was employed. Fig. 2A shows the RT-PCR products obtained from total RNA isolated from liver (L) or isolated hepatocytes (C). A product of 325 bp, putatively corresponding to the α_{1A}-adrenoceptor, was detected in liver and hepatocytes; it was clearly much more abundant in the complete tissue than in the isolated cells. Similarly a product of 481 bp was obtained when the oligonucleotides selective for the α_{1D}-adrenoceptor were used; this product was mainly observed when RNA obtained from cells, rather than from whole liver, was used. Amplification of GAPDH using RT-PCR was quantitatively similar when RNA from isolated cells or whole liver was used.

Fig. 2B shows the hybridization of the RT-PCR products against the specific α_{1A}-adrenoceptor, α_{1D}- adrenoceptor and GAPDH radiolabeled probes. The products have the expected size and they were generated from RNA, since the reactions without reverse transcriptase did not result in any band detected either by staining with ethidium bromide or by autoradiography.

In order to further corroborate the findings the fragments putatively corresponding to the α_1-adrenoceptors were cloned and sequenced. These sequences have been reported (α_{1A}-adrenoceptor, GenBank accession number AF108016 and α_{1D}-adrenoceptor, GenBank accession number AF108017). The α_{1A}-adrenoceptor guinea pig fragment showed 91% and 93.5% sequence similarity, on DNA and protein, respectively, with the human α_{1A}-adrenoceptor (GenBank accession number D25235). A similar sequence comparison of the 481 bp fragment obtained and the human α_{1D}-adrenoceptor (GenBank accession number D29952) indicated similarities of 92% and 94.3% for DNA and protein, respectively.

Experiments were performed to further substantiate the change of adrenoceptor subtype expression. Hepatocytes were isolated by collagenase digestion, as described in the Materials and methods section, and samples of the tissue were taken, at different steps, to perform RT-PCR assays. The reactions were done in the presence of 1 μCi of [α-^{32}P]dCTP and aliquots were subjected to electrophoresis, transferred onto nylon membranes and exposed to a Phospho-Screen™ for analysis. Fig. 3 shows, in the upper panel, representative autoradiographs of products corresponding to fragments of the α_{1A}-adrenoceptor, α_{1D}-

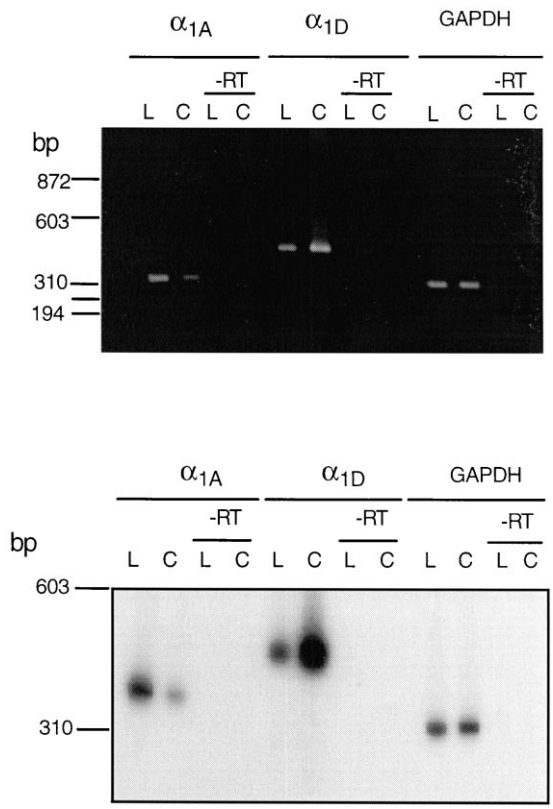

Fig. 2. Subtype-specific RT-PCR products obtained from amplification reactions using liver (L) or isolated cells (C) RNA samples. Total RNA was subjected to RT-PCR assays using the specific subtype oligonucleotides and the conditions described under the Materials and methods section. Fragments were separated on 2% agarose gels and stained using ethidium bromide (upper panel), transferred onto nylon membranes, hybridized against specific probes and subjected to autoradiography (lower panel). Assays were also performed in the absence of reverse transcriptase (-RT).

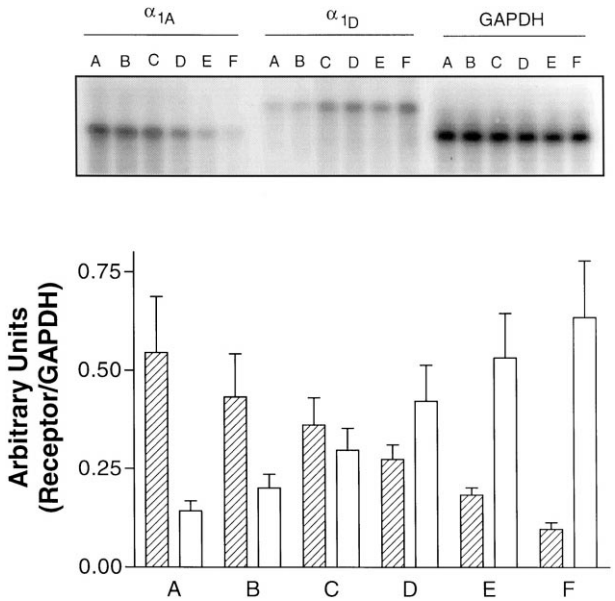

Fig. 3. Radioactive RT-PCR analysis of α_1-adrenoceptor and GAPDH fragments obtained at different stages of the hepatocyte isolation procedure. Upper panel shows representative autoradiographs of RT-PCR fragments. Samples were taken at different steps during cell isolation: (A) whole liver; (B) liver perfused for 5 min; (C) liver digested with collagenase for 10 min; (D) released cells (removal of the liver capsule and release of the cells into the washing medium); (E) cells washed and incubated in KRB buffer during 15 min at 37°C; (F) cells incubated in KRB buffer during 30 min at 37°C. Lower panel shows data for α_{1A}-adrenoceptor (dashed bars) or α_{1D}-adrenoceptor (open bars) signals normalized to GAPDH values in each condition. Plotted are the means and vertical lines represent the S.E.M. of five independent experiments.

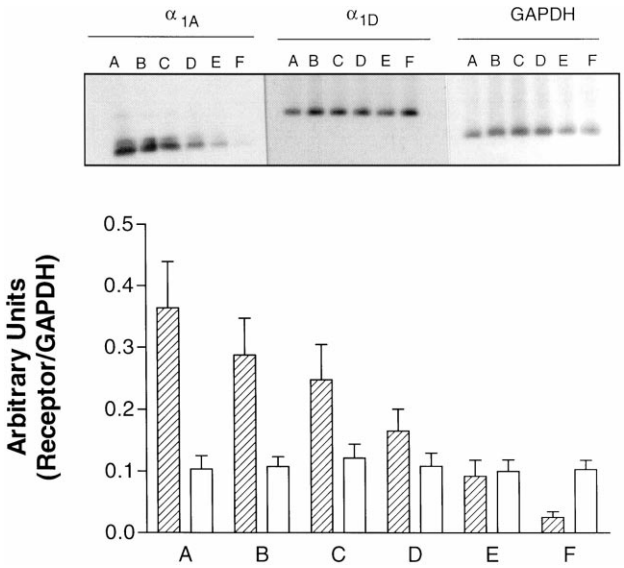

Fig. 4. Radioactive RT-PCR analysis of α_1-adrenoceptor and GAPDH fragments obtained at different stages of the hepatocyte isolation procedure from animals pre-treated with actinomycin D. Guinea pigs were injected with actinomycin D 1 h before cell isolation and actinomycin D was present during cell isolation and incubation. Other indications as in Fig. 3.

adrenoceptor and GAPDH. In the lower panel the normalized data of the expression of these receptors is presented. Consistent with previous data, it can be observed that the expression of the α_{1A}-adrenoceptor mRNA was much greater that that of the α_{1D}-adrenoceptor mRNA in whole liver and that the inverse was true for isolated liver cells. During the isolation procedure a progressive decrease in the α_{1A}-adrenoceptor signal was observed and exactly the opposite was observed for α_{1D}-adrenoceptors. The signal of GAPDH did not change during the procedure. When data were normalized considering the GAPDH signal as 1.0, it could be observed that the α_{1A}-adrenoceptor signal decreased from 0.540 in the liver to 0.093 in isolated hepatocytes, i.e., the signal decreased 6-fold. In contrast the α_{1D}-adrenoceptor signal increased from 0.141 in whole liver to 0.633 in hepatocytes incubated during 30 min, i. e., a 4.5-fold increase. The signal ratio α_{1A}/α_{1D} changed from 3.83 in the whole liver to 0.15 in the cells.

Actinomycin D was used in order to determine if the increase on the α_{1D}-adrenoceptor signal was due to induction of gene transcription. For this purpose animals were injected with actinomycin and the isolation of hepatocytes was performed also in the presence of the drug. Under these conditions, the GAPDH signal was only slightly decreased ($\sim$ 10%) at the beginning of the experiment, as compared to the signal obtained in the absence of any drug, but remained essentially constant during the isolation of hepatocytes (Fig. 4). In the presence of actinomycin the α_{1A}-adrenoceptor signal was slightly lower than in control

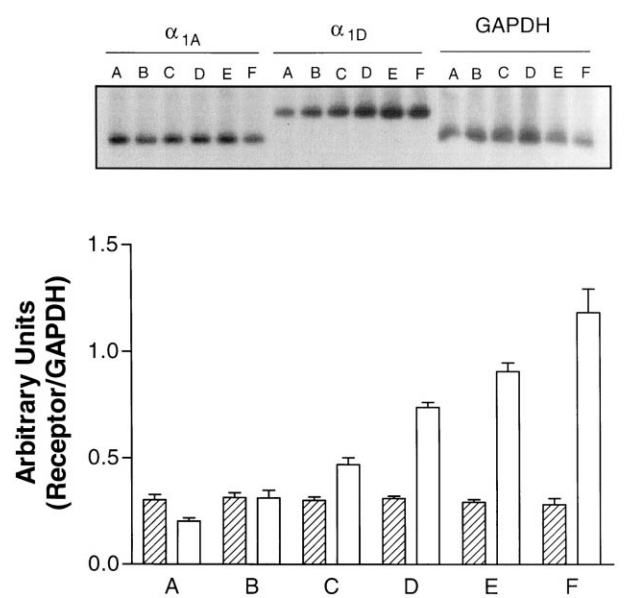

Fig. 5. Radioactive RT-PCR analysis of α_1-adrenoceptor and GAPDH fragments obtained at different stages of the hepatocyte isolation procedure from animals pre-treated with cycloheximide. Guinea pigs were treated with cycloheximide 1 h before cell isolation and cycloheximide was present during the cell isolation and incubation. Other indications as in Fig. 3.

conditions (normalized value of 0.364) and the decline of the signal was even more dramatic (to a normalized value of 0.025; i.e., a 14.6-fold decrease). Interestingly, under these conditions the signal for the α_{1D}-adrenoceptor was also slightly lower than that observed in the absence of any drug (normalized value of 0.103) and remained without change during the cell isolation procedure (normalized value 0.104). Under these conditions the signal ratio α_{1A}/α_{1D} changed from 3.53 in the whole liver to 0.24 in the cells.

Fig. 5 shows the results obtained from livers and cells isolated in the presence of the inhibitor of protein synthesis, cycloheximide. It can be observed that the initial signal for the α_{1A}-adrenoceptor (normalized value of 0.303) did not diminish during the cell isolation procedure (normalized value of 0.281). Surprisingly, the α_{1D}-adrenoceptor signal was slightly increased by the treatment at the beginning of the isolation procedure (normalized initial value of 0.202) but nevertheless it markedly increased during cell isolation (to a normalized value of 1.180; i.e., 5.84-fold increase). Under these conditions the signal ratio α_{1A}/α_{1D} changed from 1.50 in the whole liver to 0.24 in the cells.

4. Discussion

α_1-Adrenoceptors have been intensively studied in recent years and it is now clear that there is heterogeneity among this receptor subfamily and that the actions and regulation of these subtypes may differ (Theroux et al., 1996; Vázquez-Prado and García-Sáinz, 1996; Tao et al., 1997; Wenham et al., 1997). Therefore, it is of biochemical, pharmacological and therapeutic interest to know if a subtype is present in a tissue and the actions that it mediates.

As mentioned, our laboratory has reported that the livers of distinct species express different α_1-adrenoceptor subtypes. Guinea pig hepatocytes have been especially puzzling since pharmacological and radioligand binding analysis indicated the presence of α_{1A}-adrenoceptor but Northern analysis showed hybridization signal with the α_{1D} but not with the α_{1A}-adrenoceptor probes (García-Sáinz et al., 1992a,b, 1995d). In order to clarify this enigma, the technique for isolation of total RNA was improved and hybridizations were done with RNA from guinea pig liver and isolated hepatocytes. The data confirmed the results obtained in previous work about subtype-specific mRNA expressed in guinea pig hepatocytes and indicate that during the isolation of hepatocytes there is a change in expression. In other words, the subtype expressed in the liver is of the α_{1A}-adrenoceptor subtype but when the cells are isolated, the mRNA coding for this receptor is degraded and the expression of a different subtype (the α_{1D}-adrenoceptor subtype) is markedly induced by the cell isolation procedure. RT-PCR assays further substantiate this finding.

We were able to amplify fragments of the expected size and the identity was confirmed by Southern blot analysis and sequencing. Comparison of the sequences with the G-protein coupled receptor database, showed, as expected, a great similarity with the human α_1-adrenoceptor.

It has been shown in rat liver that isolation of hepatocytes under conventional conditions results in activation of the immediate-early growth program and in an extensive down regulation of most hepatic functions (Rana et al., 1994). In our study we questioned whether the change in α_1-adrenoceptor subtype gene expression was initiated early during the cell isolation procedure. To assess at which step this event occurs, we analyzed total RNA isolated from livers or hepatocytes at critical stages of the procedure. It could be observed that the transition of mRNA synthesis was initiated very early, starting at the perfusion step. The synthesis of the α_{1D}-adrenoceptor mRNA was accompanied by a symmetrical decrease in the α_{1A}-adrenoceptor mRNA expression, going from an α_{1A}/α_{1D} mRNA ratio of 3.83 in whole liver to 0.15 in hepatocytes incubated 30 min at 37°C. It is clear from the data that the changes observed mainly reflect those taking place in parenchymal cells since they were observed in the whole organ during perfusion and before cell dissociation. In fact, perfusion of the liver for 60 min resulted in an α_{1A}/α_{1D} mRNA ratio of ~ 1 (data not shown). It should be mentioned that the changes we observed were at the level of mRNA and that attempts to study functional data during culture were not possible due to a marked decrease in α_1-adrenergic responsiveness (data not shown). This has been observed in cultured rat hepatocytes (Kajiyama and Ui, 1994). In cultured guinea pig hepatocytes co-expression of α_{1A} and α_{1D} mRNA was observed (data not shown). Our data indicate that cell isolation can modulate, very rapidly, the expression of α_1-adrenoceptor subtype mRNAs. It is also clear that liver cells possess system(s) for accelerated mRNA synthesis and degradation of the adrenergic subtypes.

In order to know if differential transcription was involved in the observed phenomena, we pre-treated the animals with actinomycin D before the isolation of hepatocytes. During isolation of the cells, signal for α_{1A} was lower at each step, indicating a rapid degradation of that mRNA; however, the signal coming from α_{1D} mRNA remained constant, without any significant increase. This data indicate that the main mechanism for the α_{1D}-adrenoceptor mRNA increased expression is de novo transcription, although the possibility of a minor role of mRNA stabilization cannot be ruled out. Similarly, it is clear that transcription is not required for the rapid degradation of α_{1A}-adrenoceptor mRNA observed during cell isolation.

The data obtained with cycloheximide were interesting. This inhibitor of protein synthesis blocked the decrease in α_{1A}-adrenoceptor mRNA expression during hepatocyte isolation. It is known that cycloheximide stabilizes mRNAs putatively by blocking the synthesis of short-lived

nucleases and/or specific destabilizing factors (Peltz and Jacobson, 1992). Therefore, it is possible that cycloheximide might stabilize α_{1A}-adrenoceptor mRNA decreasing its degradation rate. Transcription could also be involved in this effect since cycloheximide induces the α_{1B}-adrenoceptor gene by activation of transcription in DDT1 MF-2 cells (Hu and Hoffman, 1993). Similarly, the very high α_{1D}-adrenoceptor mRNA signal observed during cell isolation in the presence of cycloheximide might involve stabilization of the mRNA.

Changes in adrenergic responses during isolation and culture of rat hepatic cells have been reported. It is well known that, during culture, the α_{1B}-adrenergic response rapidly decreases at the same time that a very strong β_2-adrenergic response emerges (Kajiyama and Ui, 1994). It is interesting to mention, that the changes in the expression of α_{1B}- and β_2- adrenoceptors during culture are modulated by the cell density and that addition of cell membranes to the culture may also modulate such effects (Kajiyama and Ui, 1994). Those data suggest that cell–cell interactions (i.e., yuxtacrine communication) are important in determining the type of adrenoceptor gene expressed, as shown for other genes (Rana et al., 1994). It is possible that the changes in the expression of subtypes of the α_1-adrenoceptors during cell isolation, here reported, could also be related to disruption of cell–cell interactions. However, this aspect and the possible physiological role for this change need to be directly addressed in future studies.

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