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SHORT COMMUNICATION

Drug-Metabolizing Enzymes in Rat Liver Myofibroblasts

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ABSTRACT. The myofibroblast is considered to be a key component in the pathogenesis of hepatic fibrosis. There is a need for therapeutic intervention in hepatic fibrosis, and, to date, the number of efficacious anti-fibrotic drugs is negligible. At best, the current therapeutic modalities reduce liver enzymes, an indicator of liver damage, but cannot reduce or prevent fibrosis. We have described the anti-fibrotic effect of pentoxifylline in an experimental model of hepatic fibrosis. Evidence suggests that, in addition to pentoxifylline itself, at least two of the metabolites of pentoxifylline are of therapeutic interest. We have reported that one of these metabolites (M-1) has a biological activity similar to that of its parent drug. The second metabolite (M-1R) has been reported to be more potent than the parent drug. Recent evidence suggests that inhibition of cytochrome P450 1A2 (CYP1A2) results in higher levels of pentoxifylline and M-1 and may be responsible for the production of the novel, potent metabolite (M-1R). We therefore investigated whether the myofibroblast, the cell with a crucial role in fibrosis, contains drug-metabolizing enzymes and thus may play a critical role in the anti-fibrotic actions of pentoxifylline. Our results showed that myofibroblasts contain aryl hydrocarbon hydroxylase activity, ethoxyresorufin O-deethylase activity, and methoxyresorufin O-demethylase activity. The results presented here also indicate that aryl hydrocarbon hydroxylase and methoxyresorufin O-demethylase activities can be increased by treatment of cells with dibenzanthracene, an inducer of CYP1A activities. BIOCHEM PHARMACOL **55**;5:703–708, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. myofibroblast; aryl hydrocarbon hydroxylase; ethoxyresorufin O-deethylase; methoxyresorufin O-demethylase

Hepatic drug metabolism is a process that occurs largely in the hepatocyte. Enzymes that are involved in the metabolism of drugs are also found in the Kupffer cells of the liver, and the activity of these enzymes is modified by factors that alter hepatic drug-metabolizing enzymes in hepatocytes and hepatic microsomes [1, 2]. We report here for the first time that key enzymes involved in drug metabolism are also found in the myofibroblast. The hepatic stellate cells (Ito cells, fat-storing cells) are located in the space of Disse in close contact with hepatocytes and become activated to myofibroblast cells as an integral event in fibrosis [3]. Hepatic stellate cells are cellular constituents of normal liver. The myofibroblast is a transformed hepatic stellate cell that under normal conditions is not found in the liver, but that under pathological conditions, such as hepatic fibrosis, becomes an important component of the liver cell population.

It is now well accepted that drug-metabolizing enzymes can be induced and inhibited by a number of different compounds. One group of agents that induce drug-metabolizing enzymes are the polycyclic aromatic hydrocarbons. Treatment with DBA[‖] increases the CYP1A-mediated activity of benzo(*a*)pyrene hydroxylase, EROD, and MROD [4, 5]. Studies using classical inducers have now shown that induction of drug-metabolizing enzymes occurs *in vivo* [5] and also *in vitro* in cell culture in HepG2 hepatoma cells [4], and in human hepatocyte lines from normal tissue [6].

In the present study, we investigated the possibility that myofibroblast cells have drug-metabolizing enzyme activity. Myofibroblast cells play a key role in hepatic fibrosis such that increased proliferation of myofibroblast cells and increased collagen synthesis and secretion by myofibroblast cells are the two major events in hepatic fibrosis. We reported previously that hepatocyte and Kupffer cells have measurable AHH activity and that this activity is decreased during the development of hepatic fibrosis [1]. Inhibition of drug-metabolizing enzymes occurs in hepatocytes, monocytes, and Kupffer cells [2, 7, 8]. Thus, it was important to investigate whether the myofibroblast cells contain drug-metabolizing enzymes and, therefore, could play a key role in the antifibrotic effect of pentoxifylline. Inhibition of myofibroblast CYP1A2 may facilitate accumulation and

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‖*Abbreviations:* PDGF, platelet derived growth factor; DBA, dibenzanthracene; AHH, aryl hydrocarbon hydroxylase; CYP, cytochrome P450; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; M-1, metabolite-1, 1-(5-*S*-hydroxyhexyl)-3,7-dimethylxanthine; and M-1R, 1-(5-*R*-hydroxyhexyl)-3,7-dimethylxanthine.

hence efficacy of the 800-fold more potent (but labile) metabolite (M-1R). To date, drug-metabolizing enzymes have not been studied in the myofibroblast cell; thus, this paper provides the first information available on these enzymes in this cell of hepatic origin.

MATERIALS AND METHODS

Hepatic Stellate Cell Preparation

Hepatic stellate cells were isolated from rat liver. The liver was incubated with 0.45 mg/mL collagenase type IV in Seglen's solution. Then the cells were placed on ice for 20 min, and the supernatant was removed, layered in a 15-mL centrifuge tube onto a layer of Optiprep solution [50% Optiprep (obtained from Nycomed Pharma) in 0.8% NaCl and 10 mM tricine, pH 7.8], and centrifuged at 250 *g* for 10–15 min at 4°. The cell preparation was applied to a second tube containing Optiprep (25%, 20%, 10%, 5%) and centrifuged at 1,000 × *g* for 25 min at 20°. Cells at the boundary between the 5 and 10% Optiprep layers were aspirated; these cells are the hepatic stellate cells and are characterized by spontaneous fluorescence due to their vitamin A content. After 2 weeks in culture, these cells express α smooth muscle actin, detected by immunocytochemistry. The cell preparations at this stage contained only hepatic stellate cells (positive fluorescence due to vitamin A content) and myofibroblasts (positive α smooth muscle actin immunoreactivity) and no hepatocytes or Kupffer cells. The preparation of hepatic stellate cells and subsequent culture conditions excluded the presence of hepatocytes and Kupffer cells. The hepatic stellate cells, which express α smooth muscle actin, are activated (and are called myofibroblasts); they express PDGF receptors on their cell surface, can be stimulated to proliferate with PDGF, and will synthesize collagen.

The hepatic stellate cells were isolated from Sprague–Dawley rats and initially showed the positive spontaneous fluorescence characteristic of hepatic stellate cells. The positive fluorescence declined in culture, and the decline corresponded to activation of the hepatic stellate cell to myofibroblast phenotype. Hepatic stellate cells were maintained in culture until they became activated to the myofibroblast phenotype. The myofibroblast phenotype was confirmed by positive immunohistochemistry with α smooth muscle actin antibody. The myofibroblasts were maintained in culture for several months until assayed for AHH, EROD, and MROD activities. With the long-term culture, we were assured that the cells were all of the activated phenotype. Myofibroblast cells were subcultured, plated, and allowed to become confluent. Each plate typically had between 4 and 5 × 10^6 cells. Two plates were pooled for each AHH assay; thus, 1 × 10^7 cells were used for each AHH assay, which included sample and blank readings and allowed sufficient cells for protein determination. The typical protein concentration per confluent plate was approximately 3 mg. The conditions for induction involved addition of DBA (1 or 10 μM for 48 to 72 hr). DBA was added in the standard medium, which was Dulbecco's Modified Eagle's Medium (Gibco) plus CPSR-1 10% (Sigma). The DBA was incubated with cells for 48 hr at 37°. Typically, two plates of cells were used for the AHH assay. EROD and MROD assays were carried out in triplicate or quadruplicate, using one plate of cells per reaction.

AHH Assay

The AHH assay [9] was based on the method of Cantrell and Bresnick [10]. Cells were harvested by scraping and homogenized, homogenates were incubated with benzo(*a*)pyrene and cofactors, and the 3-OH-benzo(*a*)pyrene metabolite was detected by fluorescence spectrophotometry (with a detection limit of 1 pmol). Each sample to be assayed for AHH activity contained approximately 0.5 mg protein, and the enzyme activities were expressed per milligram of protein, where protein was determined as previously described [1].

EROD and MROD Assay

To assess EROD or MROD activity, myofibroblast cells in culture were exposed for 72 hr to DBA (10 μM) in medium or to medium alone. Then myofibroblasts were incubated for 30 min with 5 μM substrate (ethoxyresorufin or methoxyresorufin) in a final volume of 5 mL at 37° in a shaking water bath. The cells (typically 4 × 10^6) and medium were harvested, and the fluorescence of the product (resorufin) was measured at emission wavelength = 600 nm and excitation wavelength = 580 nm. These assays are done under low light. Typical EROD activity has been reported to be 2.3 ± 0.1 pmol/min/mg protein for control HepG2 cells with induction to 35.6 ± 3.1 with benzanthracene (25 μM). A known concentration of resorufin (10 μL of 10 μM) was used as a standard.

Immunocytochemistry Using α Smooth Muscle Actin Antibody with Immunoperoxidase Staining

The activated hepatic stellate cells were prepared by culturing on cover slips. Positive control human intestinal smooth muscle cells (ATCC CRL 1692) were prepared in the same manner. The cover slips were washed with PBS and fixed in 10% (v/v) formalin (3.7% formaldehyde) in PBS for 40 min at room temperature. After washing three times with PBS containing 1% saponin (Sigma Chemical Co.) to permeabilize the cells, the cover slips were incubated with undiluted horse serum for 1 hr at room temperature to block non-specific staining. Then the cover slips were incubated with monoclonal anti-α smooth muscle actin (Dako Corp.) at 1:1500 for 30 min at room temperature, washed three times with PBS–0.1% saponin, and detected with goat-anti-mouse horseradish peroxidase (Dako Corp.) 1:200 for 30 min at room temperature. After washing three times in PBS, the immunochemical reaction was visualized by immersing the cover slips in 3-amino-

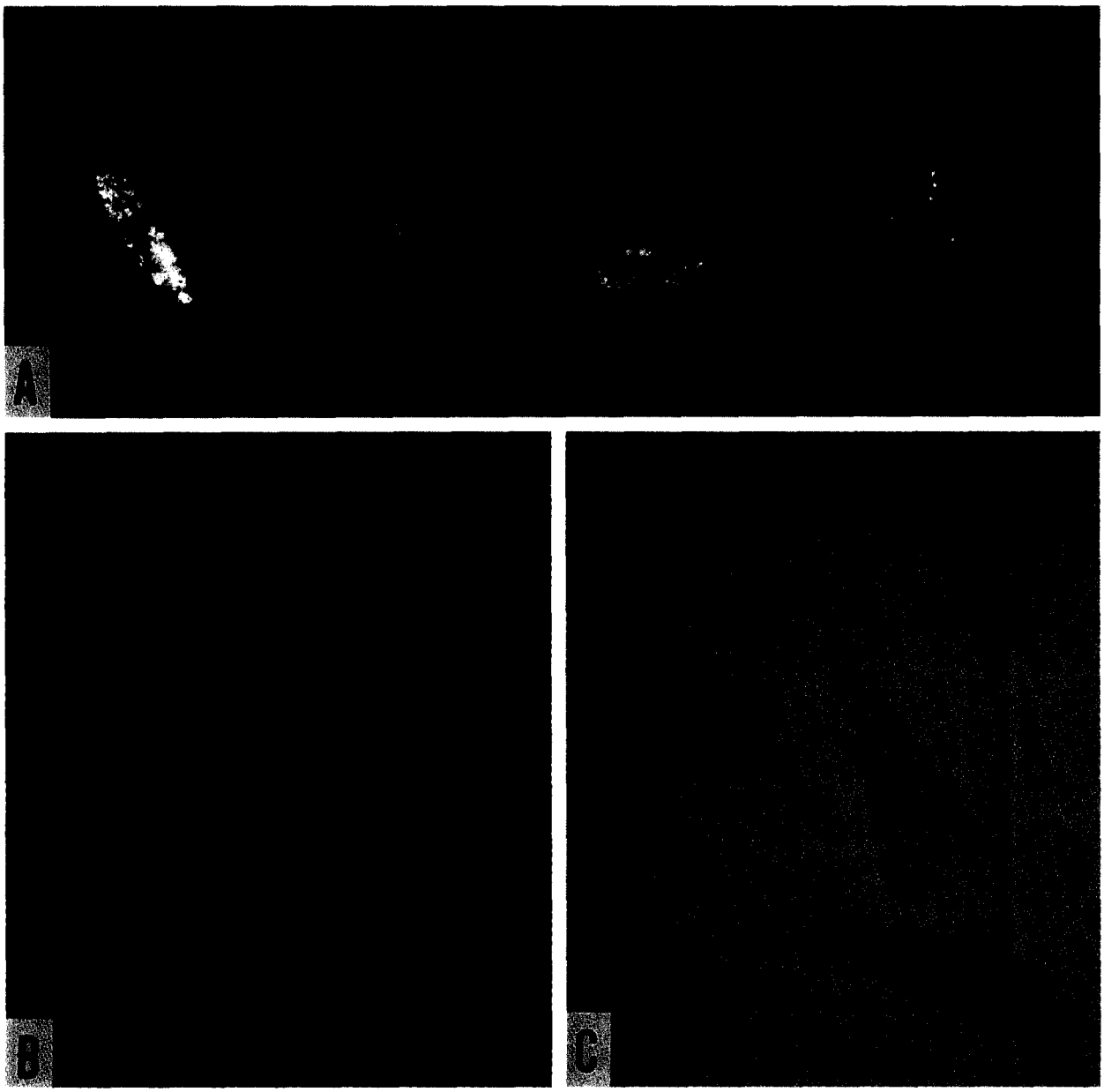

FIG. 1. (A) Spontaneous fluorescence of hepatic stellate cells. Freshly isolated hepatic stellate cells demonstrate spontaneous fluorescence due to the vitamin A content in these cells. (B) Immunocytochemical detection of α smooth muscle actin in myofibroblasts. The myofibroblasts (or activated hepatic stellate cells) demonstrate positive immunoreactivity to α smooth muscle actin antibody. Immunocytochemistry was done as described in detail in "Materials and Methods." (C) Immunocytochemical detection of α smooth muscle actin in intestinal smooth muscle cells (positive control).

ethyl-carbazole (Sigma Chemical Co.) prepared in acetate buffer (pH 5.2) containing 1% H_2O_2. The cover slips were counterstained with hematoxylin, blued with Scott's tap water, and mounted in glycerol gelatin (Sigma Chemical Co.). For negative controls, the primary antibody was omitted.

RESULTS

The quiescent hepatic stellate cell shows typical fluorescence due to vitamin A content. This phenotype is characteristic of freshly isolated cells (Fig. 1A). The cells at this stage have not differentiated and do not show reactivity with antibody to α smooth muscle actin. Following activation in culture, the myofibroblast (or activated hepatic stellate cell) phenotype was confirmed by positive immunohistochemistry with α smooth muscle actin antibody (Fig. 1B). Cells were maintained in culture until all cells demonstrated the activated phenotype. Intestinal smooth muscle cells were used as the positive control (Fig. 1C).

The results shown in Fig. 2 illustrate the AHH activity in control and DBA-induced hepatic myofibroblast cells. Bars represents the mean ± SEM of triplicate samples of hepatic myofibroblast cells obtained from three separate hepatic

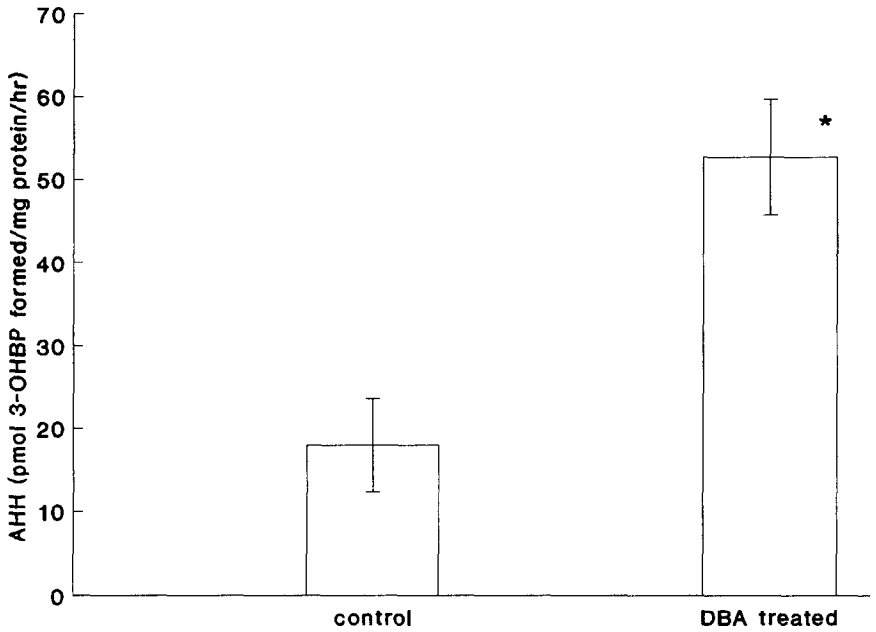

FIG. 2. AHH activity in hepatic myofibroblast cells with and without DBA treatment. AHH activity was measured in myofibroblasts as described in "Materials and Methods" and is expressed in picomoles of 3-hydroxybenzo(*a*)pyrene (3-OHBP) formed per milligram of protein per hour. Each bar represents the mean ± SEM for 9 samples. This experiment was repeated three times and gave the same results. Key: (*) significantly different ($P < 0.05$) from cells without DBA treatment.

stellate cell preparations assayed on different days for AHH activity. The results indicate that the control samples had approximately 20 pmol of AHH activity/mg protein/hr. The figure also illustrates the effect of DBA induction on hepatic myofibroblast cell AHH activity. The results indicate that the AHH activity was elevated significantly (approximately 2.5-fold) following DBA induction to an AHH activity level of 55 pmol/mg protein/hr. These results illustrate that hepatic myofibroblast cells possess AHH activity and that DBA (10 μM) for 48 hr significantly ($P < 0.05$) induced the hepatic myofibroblast AHH activity.

Figure 3 shows that EROD activity was detectable in

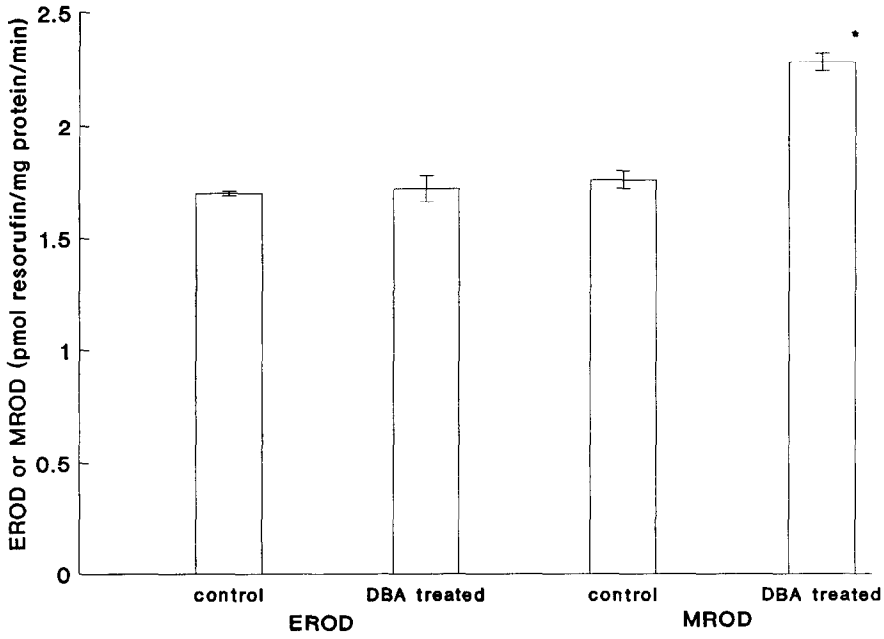

FIG. 3. EROD and MROD activities in hepatic myofibroblast cells with and without DBA treatment. EROD and MROD activities were measured in myofibroblasts as described in "Materials and Methods" and are expressed in picomoles of resorufin formed per milligram of protein per minute. Each bar represents the mean ± SEM for 6 samples. This experiment was repeated three times and gave the same results. Key: (*) significantly different ($P = 0.0001$) from cells without DBA treatment.

hepatic myofibroblast cells such that control myofibroblasts produced 1.70 ± 0.01 pmol resorufin/mg protein/min. These results were highly reproducible and showed excellent agreement between plates of myofibroblasts and between assays done on different preparations of cells assayed on different days. Exposure of myofibroblasts to DBA (10 μM) for 48 or 72 hr resulted in EROD activity (1.72 ± 0.06 pmol resorufin/mg protein/min) that was not different from controls.

Figure 3 also shows that MROD activity was detectable in hepatic myofibroblast cells such that control myofibroblasts produced 1.76 ± 0.04 pmol resorufin/mg protein/min. These results were highly reproducible and showed excellent agreement between plates of myofibroblasts and between assays done on different preparations of cells assayed on different days. Exposure of myofibroblasts to DBA (10 μM) for 72 hr significantly elevated MROD activity (2.28 ± 0.04 pmol resorufin/mg protein/min) compared with controls.

DISCUSSION

The results presented here describe, for the first time, the presence of CYP1A-mediated drug-metabolizing activity in myofibroblasts. Our recent evidence indicated that AHH activity was present in the Kupffer cells of the liver and in the monocytes isolated from peripheral blood [1, 2]. AHH activity is detectable in swine monocytes (0.32 ± 0.13 nmol/mg protein/hr), swine Kupffer cells (0.38 ± 0.21 nmol/mg protein/hr), murine Kupffer cells (0.66 ± 0.2 nmol/mg protein/hr), murine peritoneal macrophages (0.33 ± 0.1 nmol/mg protein/hr), rat monocytes (0.53 ± 0.2 nmol/mg protein/hr), and human monocytes (0.41 ± 0.06 nmol/mg protein/hr) [9].

In the present study, we assessed myofibroblast AHH, EROD, and MROD activities. Our results show that, on the average, myofibroblasts contained AHH (0.33 pmol/mg protein/min), EROD (1.7 pmol/mg protein/min), and MROD (1.75 pmol/mg protein/min) activity. Our results also indicated that the levels of AHH and MROD activity can be induced (250 and 30%, respectively) by treatment of cells with DBA. The AHH activity in myofibroblasts was equivalent to 5% of the AHH activity in Kupffer cells and less than 1% of the level of AHH activity in liver microsomes. The EROD activity in myofibroblasts was comparable to control HepG2 cells (2.3 ± 0.1 pmol/mg protein/min) [4], and to a human hepatocyte line derived from normal liver tissue (0.4 to 2.17 ± 0.44 pmol/mg protein/min) [6], and represents approximately 10% of the EROD activity present in liver microsomes (33.8 ± 10 pmol/mg protein/min). Unlike the other cells, though, EROD was not induced in myofibroblasts by treatment with DBA. The lack of effect of DBA on EROD activity may relate to the fact that these cells are of rat origin. DBA is an effective inducer of CYP1A2 as well as CYP1A1, and a recent study suggests that EROD may not be a good index of CYP1A2 activity in the rat [11]. The MROD activity in myofibroblasts was comparable to a human hepatocyte line derived from normal liver tissue (1.16 to 1.5 pmol/mg protein/min) [6], and represents approximately 5% of the MROD activity present in liver microsomes (59 ± 19 pmol/mg protein/min). Though MROD activity was increased in myofibroblasts treated with DBA, the magnitude of induction was considerably less than that observed with the human hepatocyte line, in which MROD increased to 46 ± 14 pmol/mg protein/min following induction with DBA.

Myofibroblasts from fibrotic livers are very much like the myofibroblasts that have been activated in culture. The myofibroblast, whether from fibrotic liver or activated in culture, is capable of synthesizing collagen and is also capable of marked proliferation in response to PDGF [12, 13]. In contrast, the non-activated cells in normal liver neither proliferate in response to PDGF nor synthesize vast amounts of collagen. Hepatic stellate cells, which are activated in culture to become myofibroblasts, are used as the "gold standard" for study of mechanisms in hepatic fibrosis [14].

Myofibroblast cells play a key role in hepatic fibrosis. The myofibroblast undergoes proliferation and synthesizes the collagen, which produces the fibrotic scar. We now know that these functions in this specific cell are inhibited by pentoxifylline [12, 13, 15, 16] and metabolite-1 (or M-1) [12, 15, 17], and that pentoxifylline blocks fibrosis in an animal model [18]. Inhibition of the CYP1A family of enzymes increases blood levels of pentoxifylline and the M-1 metabolite [19–21]. Inhibition of CYP1A2 will decrease the metabolism of pentoxifylline to metabolite-6 and decrease the metabolism of metabolite-1 to metabolite-7 [22], thus providing an explanation for the higher blood levels of pentoxifylline and metabolite-1 which occur when CYP1A2 is inhibited [19–21]. A novel, highly potent, labile metabolite of pentoxifylline, M-1R, is also produced under conditions whereby CYP1A2 is inhibited [23]. Thus, if this potent metabolite can be generated within the myofibroblasts, this may provide a form of localized antifibrotic therapy, i.e. production of a more potent, metabolite (M-1R), which is labile and otherwise may not achieve optimal intracellular concentrations.

The results presented here indicate that the myofibroblasts contain catalytic enzyme activities belonging to the CYP1A family. Therefore an increase in the levels of pentoxifylline and M-1 and the production of the novel M-1R in the myofibroblast (the target cell of interest for the antifibrotic effect of pentoxifylline) may play an important role in the beneficial effect of pentoxifylline in fibrosis [18], and the myofibroblast may play a critical role in maintaining the levels of this antifibrotic drug in certain forms of hepatic fibrosis [24].

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