Biochemical and Biophysical Research Communications **268,** 221–224 (2000)
doi:10.1006/bbrc.2000.2105, available online at http://www.idealibrary.com on IDEAL®

Regulation of Genes for Inducible Nitric Oxide Synthase and Urea Cycle Enzymes in Rat Liver in Endotoxin Shock

Shoko Tabuchi,*,†,[1] Tomomi Gotoh,*,[1] Kei Miyanaka,*,† Kimio Tomita,† and Masataka Mori*,[2]

**Department of Molecular Genetics and †3rd Department of Internal Medicine, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan*

Received December 27, 1999

Arginine is an intermediate of the urea cycle in the liver. It is synthesized by the first four enzymes of the cycle, carbamylphosphate synthetase I, ornithine transcarbamylase, argininosuccinate synthetase, and argininosuccinate lyase, and is hydrolyzed to urea and ornithine by arginase I, forming the cycle. In endotoxemia shock, inducible nitric oxide (NO) synthase (iNOS) is induced in hepatocytes and arginine is utilized for NO production. Regulation of the genes for iNOS and the urea cycle enzymes was studied using lipopolysaccharide (LPS)-treated rat livers. When rats were injected intraperitoneally with LPS, iNOS mRNA was markedly induced. Cationic amino acid transporter-2 and C/EBPβ mRNAs were also highly increased. In contrast, mRNAs for all the urea cycle enzymes except ornithine transcarbamylase were gradually decreased and reached 16–28% of controls at 12 h. However, all these enzymes remained unchanged at protein level up to 24 h. In light of these results, we suggest that synthesis of urea cycle enzymes is downregulated and that the protein synthetic capacity is directed to synthesis of proteins required for defense against endotoxemia.

Arginine plays a central role in the biosynthesis of urea, creatine phosphate, proline, polyamines and nitric oxide (NO) (1). In the liver of ureotelic animals, arginine is synthesized from NH_3, CO_2, ornithine and aspartate by the first four enzymes of the urea cycle, carbamylphosphate synthetase I (CPS I), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS) and argininosuccinate lyase (AL), and is hydrolyzed by arginase I to urea and ornithine, forming the cycle (Fig. 1). Under conditions of endotoxin shock, inducible NO synthase (iNOS) is induced in hepatocytes (2–7) and a large amount of NO which is important in defense reactions, is produced (8). When NO is synthesized from arginine by the iNOS reaction, citrulline, an intermediate of the urea cycle, is produced. Thus, the urea cycle is bypassed by the NOS reaction and a new cycle called "citrulline-NO cycle" is formed (Fig. 1). This citrulline-NO cycle is operative in several cell types including endothelial cells (9), macrophages (10–12) and vascular smooth muscle cells (13). On the other hand, both iNOS and arginase utilize arginine as a common substrate. We found that arginase I is markedly induced in activated macrophages, which suggested that this enzyme downregulates NO production by depleting intracellular arginine (14, 15). Arginase II (non-hepatic type) is also induced in activated macrophages (15–18), downregulates NO production (19, 20) and prevents NO-mediated apoptosis (20). Therefore, we asked if genes for arginine metabolic enzymes in the urea cycle are modulated under conditions of endotoxin shock where a large amount of NO is produced from arginine.

Here, we report that iNOS mRNA was indeed induced in the rat liver after LPS administration, whereas mRNAs for the urea cycle enzymes, except for OTC, were markedly decreased. On the other hand, mRNAs for cationic amino acid transporter-2 (CAT-2) and C/EBPβ were increased. These results suggest that synthesis of the four urea cycle enzymes is downregulated for increased synthesis of proteins which are critical in endotoxin shock.

MATERIALS AND METHODS

Animals and LPS treatment. Specific pathogen-free male Wistar rats (5–6 weeks of age) were injected intraperitoneally with *Escherichia coli* LPS (serotype 0127:B8, Sigma) at 20 mg/kg body weight, and killed at indicated times following anesthetization with ether.

RNA blot analysis. Total RNA was prepared from the rat liver by the guanidium thiocyanate-phenol-chloroform extraction procedure (21). After electrophoresis in formaldehyde-containing agarose gels, RNAs were transferred to nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized from cDNAs under the control of T7 or SP6 promoter, using a transcription kit (Boehringer Mannheim). cDNAs for the following rat proteins were used for synthesis of probes: iNOS (12), CPS I (22), OTC (22), AS (23), AL (23), arginase I (14), CAT-2 (15), C/EBPα (24) and C/EBPβ (14). Chemiluminescence signals derived from hybridized probes were detected

[1] The first two authors contributed equally to this work.

[2] To whom correspondence should be addressed at Department of Molecular Genetics, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan. Fax: 81-96-373-5145. E-mail: masa@gpo.kumamoto-u.ac.jp.

0006-291X/00 $35.00

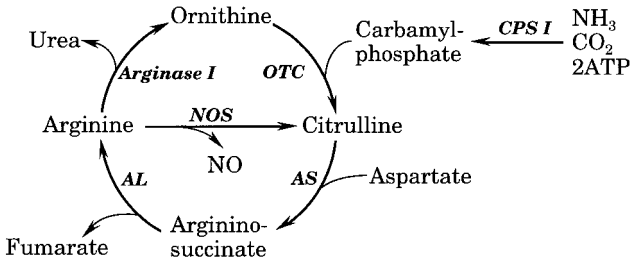

FIG. 1. The urea cycle and NO synthesis. Arginine is utilized both by nitric oxide synthase (NOS) for NO synthesis and by arginase I for urea synthesis. CPS I, carbamylphosphate synthetase I; OTC, ornithine transcarbamylase; AS, argininosuccinate synthetase; AL, argininosuccinate lyase.

on X-ray films, using a DIG luminescence detection kit (Boehringer Mannheim), then quantified using the MacBas bioimage analyzer (Fuji Photo Film Co., Tokyo, Japan).

Immunoblot analysis. Rat livers were homogenized in 9 volumes of 20 mM potassium HEPES buffer, pH 7.4, containing 0.5% Triton X-100, 1 mM dithiothreitol, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin and 50 μM pepstatin. The homogenates were centrifuged at 25,000*g* for 30 min at 4°C, and the supernatants served as liver extracts. The extracts (20 μg of protein) were subjected to SDS-PAGE, and proteins were electrotransferred to nitrocellulose membranes. For immunodetection we used an ECL kit (Amersham Pharmacia Biotech), according to the protocol provided by the manufacturer. Antibodies against rat CPS I (25), human OTC (26), rat AS (23), rat AL (23) and human arginase I (14) were used. Protein was determined using a protein assay reagent (Bio-Rad) with bovine serum albumin as a standard.

RESULTS

Changes in mRNA Levels for iNOS and Urea Cycle Enzymes in LPS-Treated Rat Liver

Rats were injected intraperitoneally with LPS and mRNAs for iNOS and the five urea cycle enzymes in the liver were measured using RNA blot analysis (Fig. 2). The amount of LPS was first titrated and the minimal dose (20 μg/g body weight) that gave nearly maximal induction of iNOS mRNA was used (data not shown). iNOS mRNA which was not detectable prior to LPS treatment, began to increase 2 h after the treatment, increased markedly at 6 h and decreased to undetectable levels within 12 h. In contrast, CPS I mRNA decreased with time up to 12 h, reached 23% of

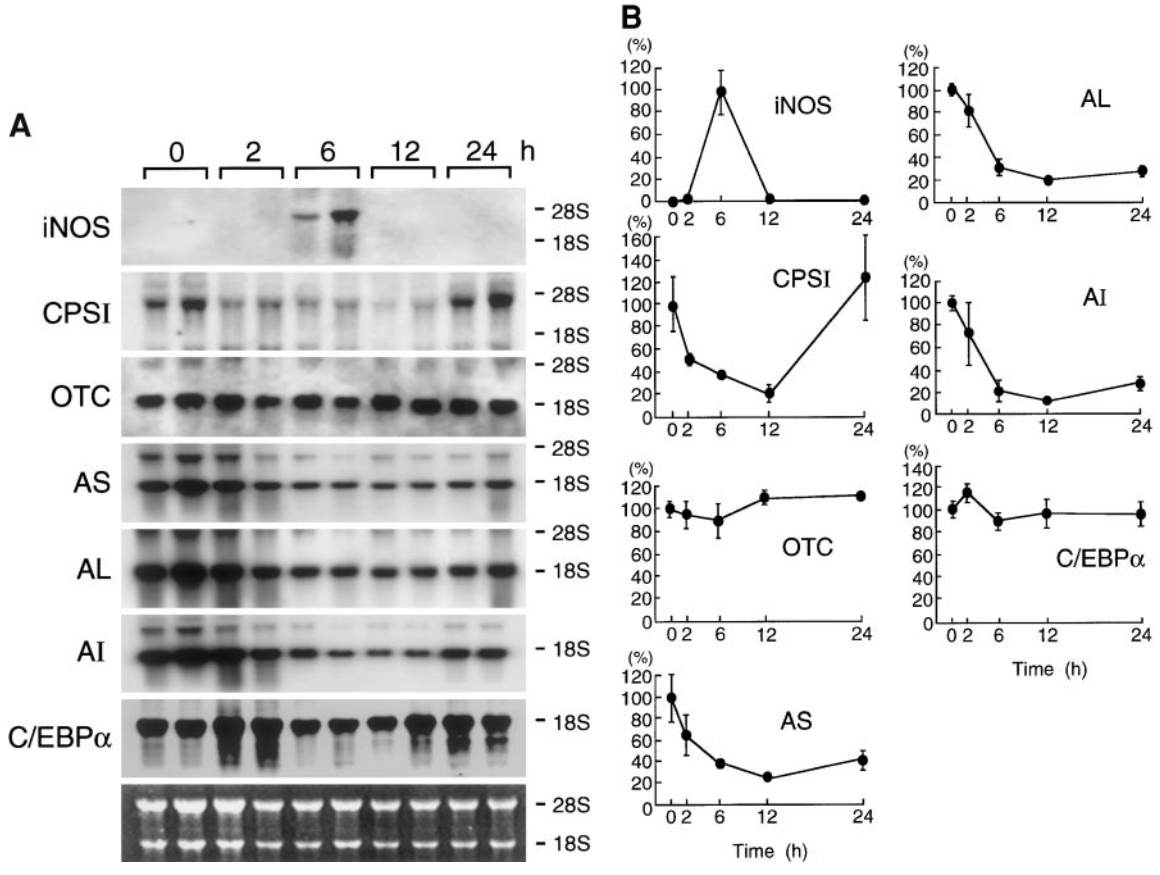

FIG. 2. RNA blot analysis of mRNAs for iNOS, urea cycle enzymes and C/EBPα in the liver of LPS-treated rats. (A) Total RNA was isolated from rat livers at the indicated times after LPS treatment. RNAs (2.0 μg) were subjected to blot analysis. The positions of 28 S (5.1 kb) and 18 S (1.9 kb) rRNAs are shown on the right. The bottom panel shows ethidium bromide staining of 28 S and 18 S rRNAs. AI, arginase I. (B) The results in A were quantified using a bioimaging analyzer and are represented by means ± ranges (n = 2). Control values at 0 time are set at 100%, except that the maximal value is set at 100% for iNOS mRNA.

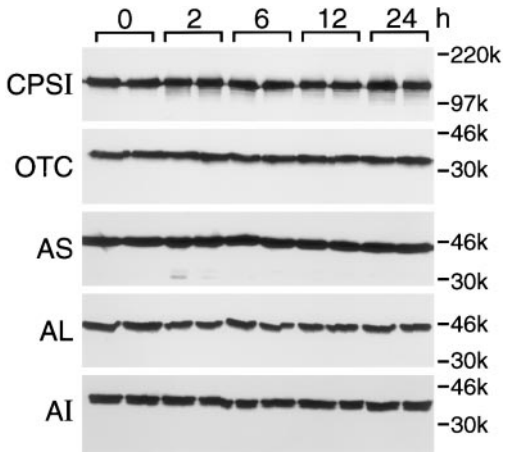

FIG. 3. Immunoblot analysis of the urea cycle enzymes in the liver of LPS-treated rats. Liver extracts were prepared at the indicated times after LPS treatment. The extracts (20 μg of protein) were applied to SDS–7% (CPS I) or 10% (OTC, AS, AL, and arginase I) polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using rabbit antisera (diluted 1:10,000) against rat CPS I, human OTC, rat AS, rat AL or human arginase I. Molecular mass markers (Rainbow protein molecular size markers, Amersham Corp.) are myosin (220 kDa), phosphorylase *b* (97 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). AI, arginase I.

control, and reverted to a level similar to or even higher than control within 24 h. mRNAs for AS, AL and arginase I also decreased up to 12 h, reaching 28%, 22% and 16% of controls, respectively, and increased somewhat at 24 h. On the other hand, OTC mRNA remained little changed for up to 24 h.

We earlier reported that expression of urea cycle enzymes, except for OTC, was strongly to moderately affected in mice deficient in transcription factor C/EBPα and showed that C/EBPα is important for liver-selected and coordinated expression of urea cycle enzymes (22). Thus, it seemed important to measure C/EBPα mRNA. However, mRNA was little changed after LPS treatment. Such being the case, the decreases in mRNAs for urea cycle enzymes may be due to a change(s) in other transcription factor(s) or to an accelerated degradation of mRNAs.

Protein Levels for the Urea Cycle Enzymes in LPS-Treated Rat Liver

Urea cycle enzyme proteins in livers of LPS-treated rats were analyzed by immunoblot analysis (Fig. 3). In contrast to mRNAs, all the urea cycle enzymes remained little changed at the protein level after LPS treatment.

Changes in mRNA Levels for CAT-2 and C/EBPβ in LPS-Treated Rat Liver

Based on these findings, we speculated that synthesis of urea cycle enzymes is downregulated in order to direct the protein synthetic capacity for synthesis of new proteins, including iNOS, which are required under conditions of endotoxin shock. We next measured mRNAs for proteins which are expected to be induced under these conditions (Fig. 4). mRNA for CAT-2 which is thought to supply arginine for iNOS reaction, was markedly induced at 2 h, decreased much at 6 h and to a level lower than control at 12 h, and returned to control level at 24 h. mRNA for C/EBPβ which activates genes for acute phase proteins in hepatocytes (27, 28), also increased at 6 h, then decreased.

DISCUSSION

In hepatocytes, activities of arginine synthetic enzymes of the urea cycle are high and a large amount of arginine is synthesized. However, the synthesized arginine is completely degraded by the high activity of arginase I, the last enzyme of the urea cycle, and there is no net synthesis of this amino acid in the liver. In endotoxin shock, iNOS is highly induced in hepatocytes and arginine is utilized for a high output production of NO. Thus arginine is utilized both by iNOS for NO synthesis and by arginase I for urea synthesis.

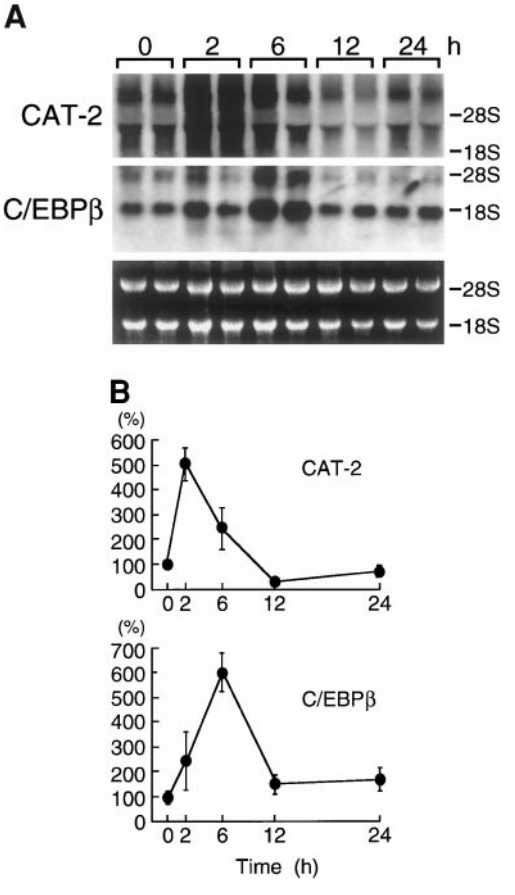

FIG. 4. RNA blot analysis of mRNAs for CAT-2 and C/EBPβ in the liver of LPS-treated rats. (A) Total RNAs (2.0 μg) were subjected to blot analysis. (B) The results in A were quantified and are represented by means $\pm$ ranges (n = 2). Control values at 0 time are set at 100%.

In the present study, we found that mRNAs for all the urea cycle enzymes, except OTC, are markedly decreased in the rat liver subjected to LPS treatment. This means that synthesis of these enzyme proteins is likely to be reduced under these conditions. Urea cycle enzymes are abundant in the liver and especially CPS I constitutes 4–5% of the total liver protein. Therefore, decreased synthesis of the urea cycle enzymes may lead to an altered profile of hepatic protein synthesis. The genes for all five urea cycle enzymes are generally regulated in a coordinated manner, but the OTC gene sometimes behaves differently from the other genes (29). We reported that C/EBPα is a key factor responsible for coordinated regulation of the urea cycle enzyme genes except OTC (22). However, C/EBPα mRNA changed little with LPS treatment. Thus, mechanisms governing decreases in mRNAs remain to be determined. In contrast, protein levels for these urea cycle enzymes remained unchanged after LPS treatment, apparently due to the slow turnover of the urea cycle enzymes. The half life of CPS I (30), OTC (31), AS (32) and arginase I (33) in adult rat livers was reported to be 7.7 days, 6–9 days, 4.4–4.6 days and 4 days, respectively.

Under conditions of endotoxin shock, proteins involved in defense functions are synthesized in the liver; expression of acute phase proteins is highly increased in the liver (34). We found that mRNAs for CAT-2 and C/EBPβ as well as iNOS are also induced. Buetler (34) found that expressions of Mn-superoxide dismutase and thioredoxin were elevated in the rat liver subjected to LPS treatment, whereas expression of glutathione S-transferase isozymes was markedly decreased; these proteins he referred to as negative acute phase proteins. The four urea cycle enzymes can be included as new members of negative acute phase proteins. All these results suggest that, in endotoxic shock, expression of genes for a group of hepatic proteins is transiently shut off and the protein synthetic capacity is directed to synthesis of a group of proteins involved in defence functions, including iNOS, CAT-2, C/EBPβ and acute phase proteins.

ACKNOWLEDGMENTS

We thank S. Oyadomari and other colleagues for discussions, M. Ohara for comments on the manuscript, and M. Imoto for secretarial services. This work was supported in part by Grants-in-Aid for Scientific Research 10557020 and 09276103 (to M.M.) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Wu, G., and Morris, S. M., Jr. (1998) *Biochem. J.* **336,** 1–17.
2. Ohshima, H., Brouet, I. M., Bandaletova, T., Adachi, H., Oguchi, S., Iida, S., Kurashima, Y., Morishita, Y., Sugimura, T., and Esumi, H. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1291–1297.
3. Geller, D. A., Nussler, A. K., Di Silvio, M., Lowenstein, C. J., Shapiro, R. A., Wang, S. C., Simmons, R. L., and Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 522–526.
4. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3491–3495.
5. Buttery, L. D. K., Evans, T. J., Springall, D. R., Carpenter, A., Cohen, J., and Polak, J. M. (1994) *Lab. Invest.* **71,** 755–764.
6. Sato, K., Miyakawa, K., Takeya, M., Hattori, R., Yui, Y., Sunamoto, M., Ichimori, Y., Ushio, Y., and Takahashi, K. (1995) *J. Leukocyte Biol.* **57,** 36–44.
7. Laskin, D. L., Rodriguez del Valle, M., Heck, D. E., Hwang, S. M., Ohnishi, S. T., Durham, S. K., Goller, N. L., and Laskin, J. D. (1995) *Hepatology* **22,** 223–234.
8. Nathan, C. (1997) *J. Clin. Invest.* **100,** 2417–2423.
9. Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E., and Vane, J. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8612–8616.
10. Wu, G., and Brosnan, J. T. (1992) *Biochem. J.* **281,** 45–48.
11. Nussler, A. K., Billiar, T. R., Liu, Z. Z., and Morris, S. M., Jr. (1994) *J. Biol. Chem.* **269,** 1257–1261.
12. Nagasaki, A., Gotoh, T., Takeya, M., Yu, Y., Takiguchi, M., Matsuzaki, H., Takatsuki, K., and Mori, M. (1996) *J. Biol. Chem.* **271,** 2658–2662.
13. Hattori, Y., Campbell, E. B., and Gross, S. S. (1994) *J. Biol. Chem.* **269,** 9405–9408.
14. Sonoki, T., Nagasaki, A., Gotoh, T., Takiguchi, M., Takeya, M., Matsuzaki, H., and Mori, M. (1997) *J. Biol. Chem.* **272,** 3689–3693.
15. Salimuddin, Nagasaki, A., Gotoh, T., Isobe, H., and Mori, M. (1999) *Am. J. Physiol.* **277,** E110–117.
16. Wang, W. W., Jenkinson, C. P., Griscavage, J. M., Kern, R. M., Arabolos, N. S., Byrns, R. E., Cederbaum, S. D., and Ignarro, L. J. (1995) *Biochem. Biophys. Res. Commun.* **210,** 1009–1016.
17. Gotoh, T., Sonoki, T., Nagasaki, A., Terada, K., Takiguchi, M., and Mori, M. (1996) *FEBS Lett.* **395,** 119–122.
18. Morris, S. M., Jr., Kepka-Lenhart, D., and Chen, L. C. (1998) *Am. J. Physiol.* **275,** E740–747.
19. Chang, C. I., Liao, J. C., and Kuo, L. (1998) *Am. J. Physiol.* **274,** H342–348.
20. Gotoh, T., and Mori, M. (1999) *J. Cell Biol.* **144,** 427–434.
21. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
22. Kimura, T., Christoffels, V. M., Chowdhury, S., Iwase, K., Matsuzaki, H., Mori, M., Lamers, W. H., Darlington, G. J., and Takiguchi, M. (1998) *J. Biol. Chem.* **273,** 27505–27510.
23. Yu, Y., Terada, K., Nagasaki, A., Takiguchi, M., and Mori, M. (1995) *J. Biochem.* **117,** 952–957.
24. Matsuno, F., Chowdhury, S., Gotoh, T., Iwase, K., Matsuzaki, H., Takatsuki, K., Mori, M., and Takiguchi, M. (1996) *J. Biochem.* **119,** 524–532.
25. Mori, M., Miura, S., Tatibana, M., and Cohen, P. P. (1981) *J. Biol. Chem.* **256,** 4127–4132.
26. Kanazawa, M., Terada, K., Kato, S., and Mori, M. (1997) *J. Biochem.* **121,** 890–895.
27. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) *EMBO J.* **9,** 1897–1906.
28. Poli, V., Mancini, F. P., and Cortese, R. (1990) *Cell* **63,** 643–653.
29. Takiguchi, M., and Mori, M. (1995) *Biochem. J.* **312,** 649–659.
30. Nicoletti, M., Guerri, C., and Grisolia, S. (1977) *Eur. J. Biochem.* **75,** 583–592.
31. Wallace, R., Knecht, E., and Grisolia, S. (1986) *FEBS Lett.* **208,** 427–430.
32. Tsuda, M., Shikata, Y., and Katsunuma, T. (1979) *J. Biochem.* **85,** 699–704.
33. Schimke, R. T. (1964) *J. Biol. Chem.* **239,** 3808–3817.
34. Buetler, T. M. (1998) *Hepatology* **28,** 1551–1560.