Augmentation of urea-synthetic capacity by inhibition of nitric oxide synthesis in butyrate-induced differentiated human hepatocytes

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Received 2 March 2000; received in revised form 3 May 2000

Edited by Veli-Pekka Lehto

Abstract We have recently developed an in vitro differentiation model of immortalized non-transformed human hepatocytes using butyrate, and observed the induction of inducible NO synthase (iNOS). In this study, we analyzed the effect of NO on the urea-synthetic capacity of these cells. The inhibition of iNOS during butyrate treatment significantly increased the urea-synthetic capacity as compared to that of butyrate treatment alone, possibly through the further induction of ornithine transcarbamylase expression. Therefore, the inhibition of NO production might be useful for obtaining more differentiated hepatocytes in the process of in vitro induction of hepatocyte-specific differentiation.

Key words: Nitric oxide; Urea; Human; Hepatocyte; Butyrate; Differentiation

1. Introduction

Nitric oxide (NO) has been identified as an important signaling molecule in the liver. Experimental results of its effect on urea-synthetic capacity, one of the most important hepatocyte-specific functions and essential for the development of an efficient bioartificial liver, are contradictory [1,2]. In animal experiments, NO has demonstrated a capacity to either reduce or increase the rate of urea synthesis of hepatocytes [1,2], implying inter-strain and inter-species differences in the NO-mediated metabolic responses [3,4]. However, this effect has not yet been evaluated in non-transformed hepatocytes of human origin.

We have recently developed an in vitro differentiation model of immortalized non-transformed human hepatocytes using butyrate, that is applicable to an artificial liver device [5,6]. Specifically, we have observed that butyrate treatment increases the urea-synthetic capacity (ammonia-detoxifying function) as well as the simultaneous growth arrest. Additionally, the expression of inducible NO synthase (iNOS) mRNA is increased in the butyrate-treated cells. The present study was conducted to investigate the mechanism of iNOS induction by butyrate and the effect of the increased NO production during the process of differentiation on the urea-synthetic capacity of these cells.

2. Materials and methods

2.1. Chemicals

Ham's F-12 medium, insulin, transferrin, hydrocortisone, L-glutamine, $CuSO_4 \cdot 5H_2O$, H_2SeO_3, $ZnSO_4 \cdot 7H_2O$, glucagon, growth hormone, linoleic acid, sodium butyrate (SB), nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), nitrate reductase from soybean seedlings, MOPS, EDTA, NADH, sulfanilamide, phosphoric acid, naphthylethylene diamine, genistein, NH_4Cl, ornithine, carbamyl phosphate, triethanolamine and citrulline were purchased from Sigma, St. Louis, MO, USA. Epidermal growth factor (EGF) was obtained form Collaborative Biomedical, Waltham, MA, USA, and sodium nitroprusside (SNP) was obtained from Merck, Darmstadt, Germany.

2.2. Cell culture

The immortalized human hepatocytes [5,6] were maintained over 150 passages in a chemically defined Ham's F-12 medium containing 50 ng/ml EGF, 10 μg/ml insulin, 5 μg/ml transferrin, 3.5 μM hydrocortisone, 2 mM glutamine, 0.1 μM $CuSO_4 \cdot 5H_2O$, 0.03 μM H_2SeO_3, 0.5 nM $ZnSO_4 \cdot 7H_2O$, 1 ng/ml glucagon, 6.5 ng/ml growth hormone and 0.5 μg/ml linoleic acid. Cells were seeded at 8×10^4/ml into each well of Primaria® multiplates (Falcon Labware, Oxnard, CA, USA) and incubated at 37°C in 5% CO_2. After 24 h, the medium was replaced by fresh medium either with 5 mM of SB, or without as a control. To inhibit iNOS, either L-NAME (3 mM) or AG (1 mM) was used. The effect of an exogenous NO donor (SNP, 0.5 mM) was also analyzed.

2.3. RT-PCR for iNOS, urea cycle enzymes and hepatocyte transcription factors

Total RNA was extracted from cells by the acid guanidinium thiocyanate–phenol–chloroform method. Complementary DNA was synthesized using 1 μg RNA and random hexamer primers. The PCR amplification of the complementary DNA was conducted in a reaction mixture containing 20 pmol of each primer set (iNOS [7], 5′-CGGTGCTGTATTTCCTTACGAGGCGAAGAAGG-3′ and 5′-GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3′; ornithine transcarbamylase (OTC) [8], 5′-GAGTTTTCAAGGGCATAGAATCGTC-3′ and 5′-CAGATCTGCTGATAGCCAT-3′; argininosuccinate synthase (ASS) [9], 5′-CACAGCCCCGAGTGTGAATTTGTCC-3′ and 5′-AGTGACCTTGCTCTGGAGACGATGA-3′; hepatocyte nuclear factor-4 (HNF-4) [10], 5′-CTGCTCGGAGCCACAAAGAGATCCATG-3′ and 5′-ATCATCTGCCACGTGATGCTCTGCA-3′; CCAAT/enhancer binding protein (C/EBP)-β [11], 5′-GCGCGAGCGCAACAACATC-3′ and 5′-TGCTTGAACAAGTTCCGCAG-3′), 200 μM deoxynucleoside triphosphates and 2 mM $MgCl_2$ in a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA). Following 2% agarose gel electrophoresis, ethidium bromide staining was performed to detect the PCR products. The number of amplification cycles for each target gene was adjusted according to the exponential amplification of each control. The intensity of each PCR product

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Abbreviations: iNOS, inducible nitric oxide synthase; SB, sodium butyrate; L-NAME, nitro-L-arginine methyl ester; AG, aminoguanidine; EGF, epidermal growth factor; SNP, sodium nitroprusside; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthase; HNF-4, hepatocyte nuclear factor-4; C/EBPβ, CCAAT/enhancer binding protein-β; NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay

PII: S0014-5793(00)01599-4

relative to that amplified using the β-actin primer set was calculated using a densitometric scanner taking the intensity of the control as 1.

2.4. Nitrite assay

Nitrite in the culture medium was measured using a modified Griess reaction following the conversion of nitrate to nitrite by nitrate reductase. Briefly, 50 μl of culture medium was mixed with 39 μl MOPS–EDTA (50 mM, pH 7.0), 10 μl NADH (2 mM), and 1 μl of 5 U/ml nitrate reductase. Following incubation for 3 h at room temperature, 100 μl of Griess reagent (0.5% sulfanilamide, 2.5% phosphoric acid, and 0.05% naphthylethylene diamine in H_2O) was added and incubated for 10 min at room temperature. Absorbance was assayed at 550 nm and compared with a standard curve obtained using sodium nitrite.

2.5. Determination of nuclear factor κB (NF-κB) activity by electrophoretic mobility shift assay (EMSA)

For this assay, cells were plated in the maintenance medium without EGF, and 24 h later, cells were treated with SB and/or EGF (50 ng/ml) for 1 h. Subsequently, nuclear extracts of cells were prepared and protein concentrations in each sample were determined using the Bio-Rad Bradford protein assay reagent. EMSA for NF-κB was performed using the Promega gel shift assay system according to the manufacturer's instructions. The effect of genistein (100 μM), an inhibitor of protein tyrosine kinases, on NF-κB activation was analyzed by treating cells for 1 h prior to and during the treatment of SB and/or EGF. A 100-fold molar excess of unlabelled oligonucleotide was used as a specific competitor.

2.6. Assay of urea synthesis

Cells previously incubated in a medium containing SB in either the presence or absence of NOS inhibitors or NO donor for 48 h were further incubated for 24 h in the same medium containing 20 mM NH_4Cl. Urea nitrogen level in the medium was colorimetrically determined at 525 nm with Sigma kit no. 535. The medium containing NH_4Cl was incubated without cells as a negative control, and tested in the same way to calculate the amount of urea synthesized. This was calculated as nmol urea nitrogen per 10^6 cells for 24 h by counting viable cells in each well.

2.7. OTC assay

Cells were harvested by scraping into mitochondria lysis buffer (0.5% Triton, 10 mM HEPES, pH 7.4, 2 mM dithiothreitol), and total protein was extracted by three freeze-thaw cycles. OTC enzyme activity was measured as previously described [12] with modifications. Briefly, 30 μg of total cellular protein was added to 700 μl of reaction mixture (5 mM ornithine, 15 mM carbamyl phosphate, and 270 mM triethanolamine, pH 7.7) which was incubated at 37°C for 30 min. Reactions were stopped by adding 250 μl of 3:1 phosphoric acid/sulfuric acid (by volume). Citrulline production was then determined by adding 50 μl of 3% 2,3-butanedione monoxime, incubating at 95–100°C in the dark for 15 min, and measuring absorbance at 490 nm.

2.8. Statistical analysis

At least three independent determinations of each parameter were compared among the treatment groups using two-sample Student's *t*-tests.

3. Results and discussion

3.1. Induction of iNOS expression and NO production

The induction of iNOS mRNA expression was evident at 6 h from the start of the butyrate treatment as compared to the control (Fig. 1A). Accordingly, butyrate treatment led to a significant increase in the concentration of nitrite in the media (Fig. 1B). This was prevented significantly by the simultaneous inhibition of NOS with inhibitors; the degree of inhibition was greater by the specific inhibitor AG than by a non-specific one, L-NAME (Fig. 1B). Exposure to an exogenous NO donor, SNP, increased the nitrite concentration significantly more than butyrate (Fig. 1B). The induction of iNOS expression in human hepatocytes by butyrate was shown for the first

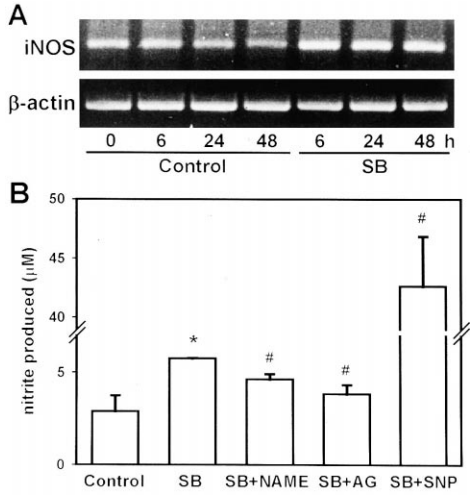

Fig. 1. Butyrate-induced increase of iNOS mRNA expression and nitrite production in immortalized human hepatocytes. A: Induction of iNOS mRNA expression by SB (5 mM), which was analyzed by RT-PCR. The figure shows one representative experiment. B: Concentrations of nitrite in media after 24-h treatment with SB in the presence or absence of NOS inhibitors or NO donor were determined by the Griess reaction. NAME 3 mM, AG 1 mM, SNP 0.5 mM. Data are expressed as the mean of three independent determinations. Bars indicate S.D. $^*P < 0.05$ compared with butyrate-untreated control cells; $^{\#}P < 0.05$ compared with butyrate-treated cells in the absence of NOS inhibitors and SNP.

time in this study, and these results are in agreement with the previous observation that iNOS expression was increased by the use of SB in Chinese hamster ovary cells which had been transfected with the human iNOS cDNA [13]. Because iNOS transcription is highly dependent on the activation of NF-κB [14], this factor was evaluated by EMSA (Fig. 2A). Given that our cells were maintained in a medium containing EGF which was previously shown to induce NF-κB [15], we evaluated the effect of EGF on NF-κB activation in parallel with that of butyrate. Butyrate-treated cells showed a shifted band of NF-κB (Fig. 2A, media 3 and 7), which was absent in experiments in which protein tyrosine kinase inhibitor genistein (Fig. 2A, media 4 and 8) or a specific competitor was added (Fig. 2A, medium 3′). No expression of iNOS mRNA was seen in response to butyrate in cells treated with genistein (Fig. 2B). In comparison, EGF-treated cells demonstrated negligible NF-κB activation when compared to control cells maintained in a medium without EGF, and did not synergistically increase NF-κB activation with butyrate (Fig. 2A, media 5 and 7). Therefore, NF-κB activated by butyrate in our cells may play a role in the induction of iNOS by butyrate.

3.2. Effects of NOS inhibition on urea-synthetic capacity

The results presented in Fig. 3 demonstrate that butyrate treatment significantly increased the concentration of urea nitrogen in medium when compared to that of the control, and that the simultaneous inhibition of NOS significantly increased the urea-synthetic capacity as compared to that of butyrate treatment alone. Moreover, in accordance with the results of nitrite analysis, urea-synthetic capacity was further increased by the treatment with AG than by L-NAME. In comparison, the butyrate-induced increase of urea-synthetic capacity was significantly attenuated by the treatment with an exogenous NO donor. These findings suggest that the in-

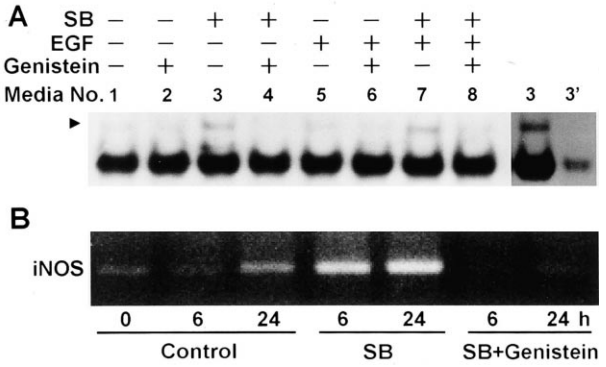

Fig. 2. NF-κB activation (A) and the effect of its inhibition on the iNOS mRNA expression (B) in butyrate-treated human hepatocytes. A: Cells were plated in the maintenance medium without EGF, and after 24 h, the medium was changed to one containing SB (5 mM) and/or EGF (50 ng/ml). Nuclear extracts of cells were prepared after 1 h, and EMSA for NF-κB was performed. The effect of genistein (100 μM) on NF-κB activation was analyzed by treating cells for 1 h prior to and during the treatment with SB and/or EGF. A 100-fold molar excess of unlabelled oligonucleotide was used as a specific competitor (medium 3′). The shifted bands of media 3 and 7 are indicated by the arrowhead. B: Induction of iNOS mRNA expression by SB, which was analyzed by RT-PCR, was abolished by the treatment with genistein.

creased production of urea by butyrate was the net result of the balance between the suppressive effect through iNOS induction and the differentiating effect as previously described [6].

L-Arginine is the substrate of iNOS. It is also the natural substrate of arginase in the urea cycle in which arginine is converted to ornithine and urea is generated. Therefore, the increase in urea production following iNOS inhibition could be due to the increased availability of substrate for arginase. However, it was recently shown that an exogenous NO donor can also reduce urea synthesis in rat hepatocytes [16], a result that was again demonstrated in human cells in this study. Therefore, NO, generated either endogenously or exogenously, may be detrimental to the urea-synthetic capacity of hepatocytes induced by butyrate.

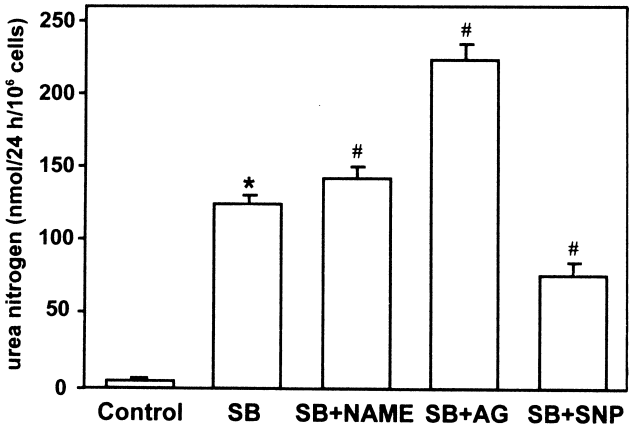

Fig. 3. Effects of SB (5 mM) in the presence or absence of NOS inhibitors or NO donor on the urea synthesis of immortalized human hepatocytes. 48 h after incubation in each indicated medium, cells were further incubated in a supplemented medium containing 20 mM NH_4Cl for 24 h. The urea nitrogen in the medium was determined colorimetrically at 525 nm. NAME 3 mM, AG 1 mM, SNP 0.5 mM. Data are represented as the mean of three independent determinations. Bars indicate S.D. $^*P < 0.05$ compared with butyrate-untreated control cells; $^\#P < 0.05$ compared with butyrate-treated cells in the absence of NOS inhibitors and SNP.

3.3. *Effects of butyrate and NO on the expression of urea cycle enzymes*

Among urea cycle enzymes, OTC and carbamyl phosphate synthetase are expressed in a tissue-specific manner in the liver and small intestine, whereas the expression of ASS and argininosuccinate lyase is not tissue-specific and more or less ubiquitous [17]. We analyzed the change of expression level of OTC and ASS, each representative of tissue-specific and non-specific ones, respectively, by RT-PCR. As shown in Fig. 4A, expression of OTC was increased by butyrate. Expression of ASS, on the other hand, was evident even before the butyrate treatment and slightly decreased after it, irrespective of NOS inhibition or exposure to an exogenous NO donor. Therefore, the induction of OTC upon differentiation by butyrate may contribute to the increased urea-synthetic capacity in these cells. The level of OTC expression further increased following simultaneous treatment with butyrate and NOS inhibitors, while that of ASS did not change as compared to that of butyrate treatment alone (Fig. 4A). Fig. 4B demonstrates that the OTC activity itself was increased after butyrate treatment and further increased by simultaneous treatment with butyrate and NOS inhibitors. Therefore, the augmentation of increase of urea synthesis by NOS inhibitors could be due to an elevated level of OTC activity. It has been shown that the mRNA level and activity of OTC, in contrast to the other four urea cycle enzymes, decrease rapidly and cannot be restored by hormones, such as glucocorticoid or

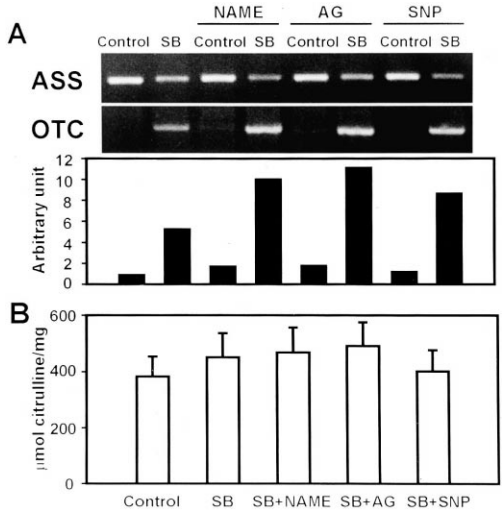

Fig. 4. Effects of SB (5 mM) treatment for 48 h in the presence or absence of NOS inhibitors or NO donor on the levels of mRNA expression of urea cycle enzymes, ASS and OTC, analyzed by RT-PCR (A) and on the activity of OTC (B). NAME 3 mM, AG 1 mM, SNP 0.5 mM. A: The arbitrary units were calculated by densitometric scanning of the intensity of each PCR product of OTC relative to that amplified using the β-actin primer, taking the intensity of the control as 1. The figure shows one representative experiment. B: OTC activity was determined by incubating total cellular protein with a reaction mixture (5 mM ornithine, 15 mM carbamyl phosphate, and 270 mM triethanolamine, pH 7.7) at 37°C for 30 min. Citrulline production was then determined by adding 3% 2,3-butanedione monoxime, incubating at 95–100°C in the dark for 15 min, and measuring absorbance at 490 nm. Data are presented as the mean of three independent determinations of OTC activity (μmol of citrulline/mg of protein). Bars indicate S.D.

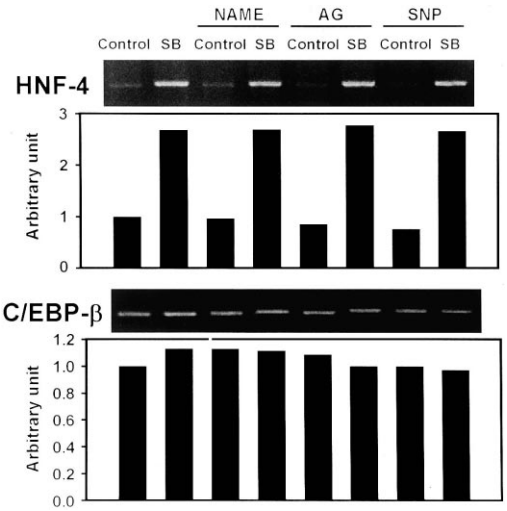

Fig. 5. Effects of SB (5 mM) treatment for 24 h in the presence or absence of NOS inhibitors or NO donor on the levels of mRNA expression of HNF-4 and C/EBPβ, analyzed by RT-PCR. NAME 3 mM, AG 1 mM, SNP 0.5 mM. The arbitrary units were calculated by densitometric scanning of the intensity of each PCR product relative to that amplified using the β-actin primer, taking the intensity of the control as 1. The figure shows one representative experiment.

glucagon, in primary cultured rat hepatocytes put into culture [18–20]. To our knowledge, the transcriptional induction of OTC as a putative mechanism underlying the increased urea-synthetic capacity of butyrate-induced hepatocytes has not been previously demonstrated.

We next analyzed whether butyrate treatment or NOS inhibition changes the expression level of the transcription factors which regulate OTC expression, such as HNF-4 and C/EBPβ [17,21], by RT-PCR. The OTC enhancer was previously shown to be activated by a combination of HNF-4 and C/EBPβ, but not by either alone [21]. Fig. 5 demonstrates that the expression of HNF-4 was increased by butyrate treatment for 24 h, while that of C/EBPβ was unchanged. Since OTC expression was not detectable after the same period of butyrate treatment (data not shown) and only became evident after 48 h (Fig. 4), this transcriptional induction of HNF-4 by butyrate is most likely to be responsible for the induction of OTC transcription. However, because simultaneous NOS inhibition did not further increase the HNF-4 expression, other mechanisms, not yet defined, may also play a role in the further increase of OTC expression by NOS inhibition. These may include the activation of HNF-4 and/or C/EBPβ by post-translational modification such as phosphorylation/dephosphorylation [22], or the decrease of truncated C/EBPβ which is a potent inhibitor of C/EBP function [23], which still remain to be determined by EMSA of these factors.

The level of OTC mRNA was also increased by treatment with an exogenous NO donor as compared to that of butyrate treatment alone (Fig. 4), while the butyrate-induced increase of urea synthesis was significantly attenuated (Fig. 3). It was previously demonstrated that NO may inhibit the hepatocyte-specific protein synthesis by affecting a translational or post-translational process [24]. Therefore, this increase of OTC mRNA level by an exogenous NO donor is likely to be due to the stabilization of its mRNA resulting from the translational inhibition, which needs to be further clarified by nuclear run-on assay. Alternatively, since NO can directly inhibit enzyme activities by interaction with SH- or metal-containing groups [25], it is also plausible that the inhibition of urea-synthetic capacity by NO may have resulted from the direct inhibition of the activity of urea cycle enzymes.

In conclusion, increased NO production in the differentiation process of human hepatocytes by butyrate may play a role in suppressing butyrate-induced urea-synthetic capacity. Therefore, the inhibition of NO production in the process of in vitro induction of hepatocyte-specific differentiation could be a reasonable way to acquire more applicable hepatocytes for the development of an efficient bioartificial liver.

Acknowledgements: The authors thank Ms. Hyejung Jung and Jinkyoung Jung for their excellent technical assistance. This work was supported by a grant from the Korean Foundation of Liver Research.

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