

β_2 -Adrenergic receptor-selective agonist clenbuterol prevents Fas-induced liver apoptosis and death in mice

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André, Claudine, Dominique Couton, Jesintha Gaston, Loubna Erraji, Laurent Renia, Paule Varlet, Pascale Briand, and Jean-Gérard Guillet. β_2 -Adrenergic receptor-selective agonist clenbuterol prevents Fas-induced liver apoptosis and death in mice. *Am. J. Physiol.* 276 (*Gastrointest. Liver Physiol.* 39): G647–G654, 1999.—Stimulation of the cAMP-signaling pathway modulates apoptosis in several cell types and inhibits Jo2-mediated apoptosis in cultured rat hepatocytes. No information is yet available as to whether the hepatic β_2 -adrenergic receptor (AR) expression level, including β_2 -AR-dependent adenylyl cyclase activation, modulates hepatocyte sensitivity to apoptosis in vivo or whether this sensitivity can be modified by β_2 -AR ligands. We have examined this using C57BL/6 mice, in which hepatic β_2 -AR densities are low, and transgenic F28 mice, which overexpress β_2 -ARs and have elevated basal liver adenylyl cyclase activity. The F28 mice were resistant to Jo2-induced liver apoptosis and death. The β -AR antagonist propranolol sensitized the F28 livers to Jo2. In normal mice clenbuterol, a β_2 -AR-specific agonist, considerably reduced Jo2-induced liver apoptosis and death; salbutamol, another β_2 -AR-selective agonist, also reduced Jo2-induced apoptosis and retarded death but with less efficacy than clenbuterol; and propranolol blocked the protective effect of clenbuterol. This indicates that the expression level of functional β_2 -ARs modulates Fas-regulated liver apoptosis and that this apoptosis can be inhibited in vivo by giving β_2 -AR agonists. This may well form the basis for a new therapeutic approach to diseases involving abnormal apoptosis.

β -adrenoceptor antagonist; C57BL/6 mouse; transgenic F28 mouse; anti-Fas monoclonal antibody

APOPTOSIS or programmed cell death is of great importance in normal development, homeostasis, and pathogenic processes. Diseases such as acquired immunodeficiency syndrome (AIDS), neurodegenerative diseases, ischemia, and hepatitis are characterized by excessive apoptosis, whereas carcinogenesis, autoimmune diseases, and viral infection are associated with insufficient apoptosis.

Apoptosis is mainly triggered by the interaction of Fas-L and tumor necrosis factor (TNF)- α (6, 13, 19, 25) with their receptors at the cell surface. Fas-L-mediated apoptosis seems to play a key role in the development of hepatitis (19). Hence, mice given the anti-Fas monoclonal antibody (MAb) Jo2 develop a phenotype of fulmi-

nant hepatitis and die (33). Several factors are potent in vivo inhibitors of Fas-mediated apoptosis in the liver. For instance, the destruction of the livers by Jo2 MAb is inhibited in transgenic mice that overexpress human Bcl-2 (22, 35), SV40 T antigen (39), or a nontransforming SV40 T-antigen mutant (38) in the liver or mice given interleukin 1 β -converting enzyme-like protease inhibitors (36, 40), hepatocyte growth factor (20), or the immunomodulator linomide (34). In all animals but one (35), the lethal effect of anti-Fas MAb was also either retarded (39) or inhibited (20, 22, 34, 36, 38, 40).

cAMP is a modulator of apoptosis in many cells in culture. Hence, increases of the intracellular cAMP concentration and/or stimulation of the cAMP-signaling pathway with cAMP analogs can inhibit apoptosis in bone marrow-derived cells (3), T-cell hybridomas (14, 24), neutrophils (37), and macrophages (29) and can induce apoptosis in myeloid leukemia cells (41), malignant glioma cells (7), T cells (18, 28), B lymphocytes (26), and ovarian granulosa cells (1). cAMP also protects rat hepatocytes in culture against bile acid-induced and Fas-mediated apoptosis (11, 44). Adenylyl cyclase activity, and thus intracellular cAMP production, can be stimulated by activating several membrane-bound receptors via interaction with heterotrimeric GTP-binding proteins. One of these receptors, the β_2 -adrenergic receptor (AR), mediates glycogenolysis and gluconeogenesis in the liver (16).

There is no information as to whether the hepatic β_2 -AR expression level, including β_2 -AR-dependent adenylyl cyclase activity and cAMP production, modulates the sensitivity of the hepatocytes to apoptosis in vivo or whether this sensitivity can be modified by giving β_2 -AR ligands. The present study examines this question using C57BL/6 mice, in which the hepatic density of β_2 -ARs is low, and recently described transgenic F28 mice, whose hepatic β_2 -AR densities are much higher and mimic those reported for humans (2).

MATERIALS AND METHODS

Animals. F28 transgenic mice were produced using the untransformed complete 3458-bp genomic fragment isolated from the human epidermoid cell line A-431 (10) as previously described (2). Control C57BL/6 mice were from Iffa Credo (Lyon, France). The studies were done on 6- to 10-wk-old (16–21 g) males. The animals were killed by cervical disruption. All animal procedures were conducted in accordance with French government regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture).

Particulate fraction preparation. Livers were homogenized for 10–20 s in a Polytron mixer and then in a Potter

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homogenizer (5 strokes, 2,000 rpm) in 50 mmol/l Tris·HCl, pH 7.6, 5 mmol/l EDTA, 250 mmol/l sucrose, 10 µg/ml benzamidine, 5 µg/ml trypsin inhibitor, 5 µg/ml leupeptin, and 10 mmol/l phenylmethylsulfonyl fluoride (PMSF) at 4°C. The homogenate was centrifuged for 20 min at 50,000 g, and the resulting particulate fraction was dispersed in 75 mmol/l Tris·HCl, pH 7.6, 5 mmol/l EDTA, 12.5 mmol/l MgCl₂, 10 µg/ml benzamidine, 5 µg/ml trypsin inhibitor, 5 µg/ml leupeptin, and 10 mmol/l PMSF supplemented with 10% glycerol and stored at -80°C. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Determination of β -AR binding characteristics and functionality on liver particulate fractions. Total β -AR-binding, β_2 -AR-binding, and adenylyl cyclase activities were determined as described earlier (2). Incubations for adenylyl cyclase assays were done in the presence of 10 µmol/l of *l*-isoproterenol, 100 µmol/l 5'-guanylylimidodiphosphate (Gpp[NH]p), or 1 mmol/l forskolin to determine maximal β -AR agonist-induced, maximal G protein-induced, or maximal non-receptor-mediated stimulation of adenylyl cyclase, respectively. Isoproterenol, Gpp[NH]p, and forskolin were from Sigma Chemical (St. Louis, MO).

Quantification of cAMP in perfused and nonperfused livers. The liver lobes were carefully removed from newly killed mice. Lobes used for experiments on nonperfused livers were immediately frozen in liquid nitrogen and kept at -80°C until the extraction of cAMP.

The functionality of the β -AR system in the intact liver was assayed *ex vivo* by perfusing isolated lobes at a flow rate of 5 ml/min, first for 2 min with 100 mmol/l HEPES, 145 mmol/l NaCl, 2 mmol/l KCl, and 0.3 mmol/l Na₂HPO₄, pH 7.6, containing 1 mmol/l IBMX (Sigma Chemical) (HI buffer) or HI buffer containing the β -AR antagonist propranolol (10 mmol/l) and then for 2 min with the same perfusion mix containing the β_2 -AR agonist clenbuterol (0.1 mmol/l). The lobes were incubated for an additional 13 min in the final perfusion solution, frozen in liquid nitrogen, and stored at -80°C until extraction of cAMP. The perfusion solutions were kept at 37°C.

cAMP was extracted from ~200 mg of tissue homogenized in 800 µl of water containing 1 mmol/l IBMX with a Potter homogenizer. A sample of the homogenate was taken for measurement of the protein content with the BCA protein assay kit. The remainder was heated in a boiling water bath for several minutes to coagulate the proteins and centrifuged for 10 min at 14,000 rpm, and the cAMP in the supernatant was assayed by a [³H]cAMP assay system (Amersham, Arlington Heights, IL). The concentration was expressed as picomoles of cAMP per milligram of homogenate protein.

Western blotting. Western blot analysis of Fas in liver particulate fractions of young adult control and F28 mice was essentially done as described previously (35). Briefly, samples containing 100–150 µg of protein in Laemmli sampling buffer were heated for 5 min at 94°C and run on 12.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then blocked with 5% nonfat milk in PBS containing 0.25% Tween (PBS-T) for 1 h. The membranes were incubated overnight at 4°C with 0.5 µg/ml hamster anti-mouse Fas antigen MAb (Jo2; PharMingen, San Diego, CA) in 2.5% nonfat milk in PBS-T, washed in PBS, and incubated with peroxidase-conjugated goat anti-hamster IgG (Jackson ImmunoResearch Labs, West Grove, PA) (0.8 µg/ml in PBS-T) for 1 h at room temperature. Finally, the membranes were washed with PBS, incubated in enhanced chemiluminescence (ECL) detection reagents (Amersham) for 1 min at room temperature, and exposed to Hyperfilm ECL (Amer-

sham). Full-range molecular weight markers (Amersham) were used to determine apparent molecular weights.

Treatment of mice and Jo2 MAb injection. The mice were given β -adrenergic agonists or antagonists in drinking water. Clenbuterol (1.3 or 2.6 mg/l; Sigma Chemical) or salbutamol (1.4 or 2.1 mg/l; Salbumol, Glaxo Wellcome) was given for ~17 h, and propranolol (14 or 21 mg/l; Avlocardyl, Zeneca-Pharma) was given for 48 h. The inhibition of the clenbuterol effects by propranolol was examined in mice first given propranolol (14 mg/l) for 48 h and then propranolol (14 mg/l) plus clenbuterol (2.6 mg/l) for 17 h. All treatments were started at 4:00–6:00 PM. The monoclonal antibody Jo2 was given by intravenous injection (15 µg/200 µl in 0.9 g/l NaCl).

Histology. Livers were removed 5–6 h after Jo2 MAb injection or immediately after death, whichever occurred first. Fragments of tissue were fixed in 4% formaldehyde and embedded in paraffin. Sections (5 µm) were cut and stained with hematoxylin-eosin.

Incidence of apoptosis. The incidence of apoptosis in the mice in each study group shown in Figs. 2, 4, 5, and 7 was summarized as described earlier (23): hematoxylin-eosin-stained liver sections were prepared for all animals used, and the cells undergoing apoptosis (chromatin condensation, nuclear fragmentation, extensive surface blebbing) were scored. The number of apoptotic bodies was counted in four fields, each of ~100 cells.

In situ detection of apoptosis. The transferase UTP nick-end labeling procedure was used. Sections were incubated in 0.1 mol/l HCl containing 50 U/ml pepsin for 5 min, washed (3 × 5 min) in distilled water, and incubated in CC buffer (140 mmol/l sodium cacodylate, pH 7.2, 1 mmol/l CoCl) for 10 min. The sections were then incubated in CC buffer containing 0.25 U/µl terminal deoxynucleotidyl transferase and 10 µmol/l bio-11-dUTP (Amersham) for 60 min in a water-saturated atmosphere and washed at room temperature once with 2 × SSC (1 × SSC is 0.15 mol/l NaCl and 0.015 mol/l sodium citrate, pH 7.0) for 10 min and twice with 100 mmol/l Tris·HCl, pH 7.6 for 3 min. Endogenous alkaline phosphatases were eliminated by incubation in 2% levamisole in PBS (Sigma) for 10 min, and the sections were washed with 100 mmol/l Tris·HCl, pH 7.6. They were then incubated with alkaline phosphatase-streptavidin in 100 mmol/l Tris·HCl, pH 7.6 (1:1,000; Vector Laboratories, Burlingame, CA) for 30 min, washed twice in 100 mmol/l Tris·HCl, pH 7.6, for 3 min and twice in 100 mmol/l Tris·HCl, pH 9.4, for 5 min, and incubated in the dark with NBT-BCIP, as indicated by the manufacturer (GIBCO BRL, Gaithersburg, MD) for 10 min. Finally, they were washed with distilled water and counterstained with methyl green. All manipulations were done at room temperature except the incubation with 10 µmol/l bio-11-dUTP, which was at 37°C.

Serum analysis. Biochemical parameters of the serum [alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT)] were quantified using a standard clinical automatic analyzer (type 917, Hitachi).

Statistical analysis. Results are presented as means ± SE. The significance of differences between means of two groups was assessed by using the Student's *t*-tests for unpaired variates and that between more than two groups by using ANOVA, which was followed, when significant, by the Bonferroni test.

RESULTS

β -AR expression and functionality in livers of F28 and control mice. We monitored the total β -AR and

β_2 -AR binding capacities as well as the basal, maximal isoproterenol-induced, maximal G protein-induced, and maximal non-receptor-mediated adenylyl cyclase activity in particulate fractions that were prepared from livers of 8-wk-old mice. The F28 mice had a 17-fold greater β_2 -AR density, an 11-fold greater maximum agonist-induced adenylyl cyclase response, and a slightly higher basal and maximum G protein-induced adenylyl cyclase activity than the control mice. The maximal activity of the adenylyl cyclase enzyme was similar for both groups (Table 1). We subsequently evaluated the extent to which β -AR agonists can modulate β -AR-dependent adenylyl cyclase activity and cAMP accumulation in the livers of F28 and C57BL/6 mice in vivo.

We first measured cAMP in livers taken from F28 and C57BL/6 mice that were untreated or treated with the β_2 -AR agonist clenbuterol (Fig. 1A). The hepatic cAMP levels in untreated and treated C57BL/6 mice did not differ statistically. Untreated F28 mice also had the same levels as untreated C57BL/6 mice. However, the cAMP levels in clenbuterol-treated F28 mice were slightly but significantly higher, indicating that the hepatic β_2 -AR system of these mice is activated by oral administration of the ligand. Because these experiments were done in the absence of phosphodiesterase blockers, the accumulated cAMP was rapidly metabolized. The slightly increased cAMP levels in the clenbuterol-treated F28 mice thus probably resulted from a considerably increased production of intracellular cAMP. It is therefore also probable that smaller increases, such as those expected to occur in clenbuterol-treated C57BL/6 mice, were not detected in these experimental conditions.

We therefore studied next the production of cAMP under stimulating and nonstimulating conditions in livers that were treated with IBMX by ex vivo perfusion (Fig. 1B). The results were in accordance with those for the particulate fractions (Table 1). Already in nonstimulating conditions, the livers of F28 mice accumulated significantly larger amounts of cAMP (5.1 ± 0.2 pmol/mg protein) than the livers of control mice (2.99 ± 0.05 pmol/mg protein). After stimulation with clenbuterol,

Table 1. Hepatic β -AR binding characteristics and functionality in C57BL/6 and F28 mice

	C57BL/6	F28
<i>Binding capacity, fmol/mg protein</i>		
β -AR	25 ± 3	$264 \pm 14^{**}$
β_2 -AR	15 ± 4	$264 \pm 6^{**}$
<i>Adenylyl cyclase activity, pmol·mg⁻¹·min⁻¹</i>		
Basal + GTP	1.9 ± 0.4	$4.3 \pm 0.6^{**}$
Isoproterenol	2.7 ± 0.3	$30 \pm 1^{**}$
Forskolin	38 ± 2	38 ± 1^{ns}
Gpp[NH]p	11.4 ± 0.9	$25 \pm 1^{**}$

Values are means \pm SE of data from triplicate assays performed on 3 different particulate fraction preparations from C57BL/6 and F28 mouse livers. AR, adrenergic receptor; Gpp[NH]p, 5'-guanylylimidodiphosphate. Statistical difference between means obtained for C57BL/6 and F28 mice is indicated. $^{**} P < 0.02$; $^{ns} P > 0.05$.

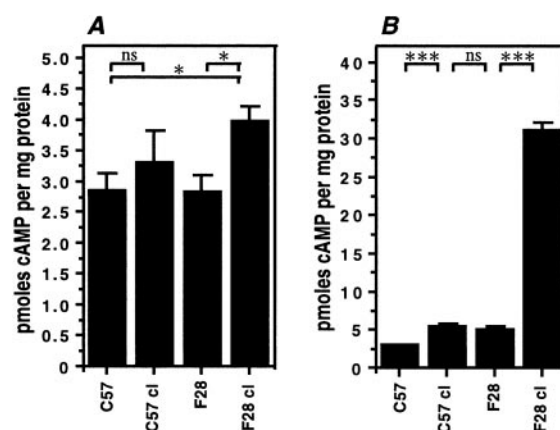


Fig. 1. Quantification of cAMP in untreated and clenbuterol-treated C57BL/6 and F28 mice. cAMP content was determined in livers of C57BL/6 and F28 mice given (C57cl, F28cl) or not given (C57, F28) 2.6 mg/l clenbuterol in drinking water (A) or in livers of untreated C57BL/6 and F28 mice treated ex vivo first for 2 min with 1 mM IBMX and then for 15 min with 1 mM IBMX (C57, F28) or 1 mM IBMX plus 0.1 mM clenbuterol (C57cl, F28cl) by ex vivo perfusion (B). Values are means \pm SE of values (pmol cAMP per mg tissue protein) obtained for 3–6 mice with 2 individual determinations for each. $^{***} P < 0.001$; $^{*} P \leq 0.05$; $^{ns} P > 0.05$.

the difference was over sixfold (31.1 ± 0.8 pmol/mg protein for F28 mice and 5.4 ± 0.3 pmol/mg protein for controls). These experimental conditions also allowed us to show that control mice livers with only a few β_2 -ARs were stimulated by clenbuterol to increase cAMP accumulation from 2.99 ± 0.05 to 5.4 ± 0.3 pmol/mg protein. The amounts of cAMP in F28 livers under nonstimulating conditions (5.1 ± 0.2 pmol/mg protein) were not significantly different from those in clenbuterol-treated livers of control mice (5.4 ± 0.3 pmol/mg protein).

F28 mice are spontaneously protected against Jo2-mediated liver apoptosis and lethality. Control and F28 mice were injected intravenously with 15 μ g of anti-Fas MAb. All the F28 mice survived, whereas $\sim 75\%$ of the control mice died within 5 h and only 1 of 20 survived for >24 h (Fig. 2B). The livers taken from the control mice showed extensive hepatocyte apoptosis (Fig. 3A), with chromatin condensation, nuclear fragmentation, extensive surface blebbing, acidophilic Councilman bodies (Fig. 4C, inset), and DNA fragmentation (Fig. 4D) and had severe intraparenchymatous hemorrhages (Fig. 4C). This is consistent with previous work (33). In contrast, the livers from F28 mice were largely unaltered 5 h after injection, with very few apoptotic foci (Figs. 5, C and D, and 3B). Accordingly, the serum aminotransferase (ALAT and ASAT) activity 3 h after the injection of Jo2 increased markedly in the C57BL/6 mice but remained very low in the F28 mice (Fig. 6).

The resistance of the F28 mice to Fas-induced apoptosis could be caused by a large decrease in Fas at the cell surface. This was checked by Western blotting with Jo2 on liver particulate fractions from F28 and control animals. Extracts from both animals gave similar signals at 45–50 kDa (Fig. 2A), corresponding to the apparent molecular mass of the Fas receptor (33, 35).

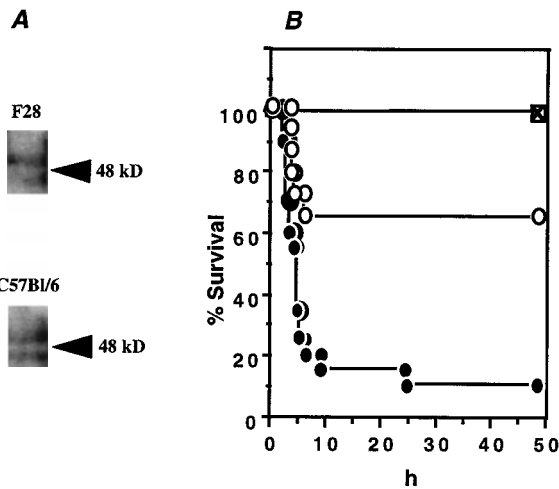


Fig. 2. A: Western blot analysis of C57BL/6 and F28 mice liver extracts. Hamster anti-mouse Fas Jo2 monoclonal antibody (MAb) and peroxidase-conjugated goat anti-hamster IgG were used to detect Fas antigen. Molecular mass markers indicated on right. B: effect of β_2 -AR-selective agonist clenbuterol on death rate of C57BL/6 (filled and open circles) and F28 (squares and crosses) mice injected iv with 15 μ g Jo2 MAb. Results shown are for 3–7 sets of experiments performed on groups of 3 mice that were untreated (filled circles and squares) or given 1.3 mg/l clenbuterol in drinking water (open circles and crosses). Data are percentages of injected mice that survived.

Antagonist propranolol decreased resistance of F28 mice to Jo2 MAb. Because F28 mice with overexpressed hepatic β_2 -ARs are naturally protected against Jo2 MAb, we examined the response of these mice to Jo2 MAb when the β -ARs were blocked with the β -AR antagonist propranolol. F28 mice were given 21 mg/l propranolol for 48 h and then injected with 15 μ g of Jo2 MAb. None of the pretreated F28 mice died, but they all had significantly more apoptotic cell bodies than did

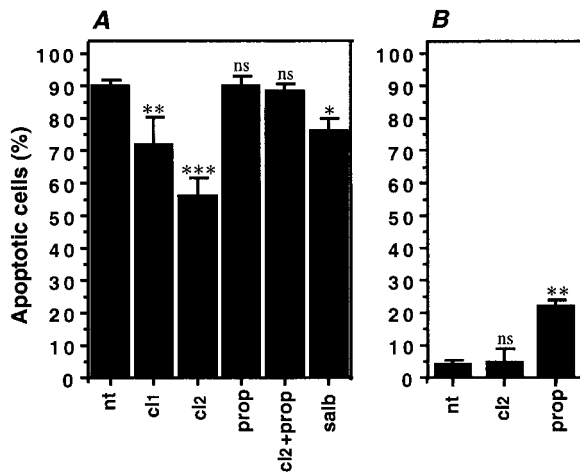


Fig. 3. Incidence of apoptosis in livers of treated and untreated mice injected with Jo2. Percentage of cells having apoptotic morphological changes was determined for all animals studied in experiments illustrated by Figs. 2, 4, 5, and 7, i.e., C57BL/6 (A) or F28 (B) mice given no treatment (nt), 1.3 and 2.6 mg/l clenbuterol (cl1, cl2), 1.4 mg/l propranolol (prop), 2.6 mg/l clenbuterol plus 1.4 mg/l propranolol (cl2+prop), or 2.1 mg/l salbutamol (salb). Data shown are means \pm SE. Comparison is made with data obtained for untreated F28 and C57BL/6 mice, respectively. *** $P < 0.001$; ** $P < 0.01$; * $P \leq 0.05$; ns $P > 0.05$.

untreated F28 mice given Jo2 MAb (Figs. 5, G and H vs. C and D, Fig. 3B). The percentage of apoptotic cells for the propranolol-treated F28 mice nevertheless remained much lower than that for untreated or even clenbuterol-treated C57BL/6 mice (Fig. 3, B vs. A), and serum levels of aminotransferase 3 h after the injection of Jo2 remained as low in propranolol-treated as in untreated F28 mice (Fig. 6).

β_2 -AR-selective agonist clenbuterol inhibited Jo2-induced lethality and liver apoptosis in C57BL/6 mice. We attempted to protect control mice against Fas-induced hepatic apoptosis by giving β_2 -AR agonists to activate the hepatic β_2 -ARs. C57BL/6 mice were given 1.3 mg/l of the β_2 -AR-selective agonist clenbuterol in drinking water for 17 h and then injected with 15 μ g Jo2 MAb. The clenbuterol-treated C57BL/6 mice had considerably less liver apoptosis than the untreated animals (Fig. 4, E and F vs. C and D; Fig. 3A). About 63% of the agonist-treated control mice were protected from the lethal effect of the Jo2 MAb (Fig. 2B). Clenbuterol-treated F28 mice also had greater natural resistance to Jo2-induced hepatocyte apoptosis than did untreated F28 mice and often had no more apoptotic cells (Fig. 5, E and F vs. C and D; Fig. 3B). Despite less severe liver injury in the C57BL/6 mice treated with 1.3 mg/l clenbuterol, the biochemical sequelae were still large and only the serum ASAT activity was significantly different from that of the untreated mice. Serum aminotransferase activity was very low in clenbuterol-treated F28 mice (Fig. 6).

Effect of clenbuterol in C57BL/6 mice was dose and β_2 -AR dependent. The dose dependence of the clenbuterol effect was assessed by giving control mice drinking water with 1.3 or 2.6 mg/l clenbuterol before injection of Jo2 MAb. One-half of the mice given the low dose died within 6 h, but all the mice given the high dose survived (Fig. 7A). The high-dose mice had fewer apoptotic hepatocytes (Fig. 3A) and significantly lower serum aminotransferase activity than the low-dose mice (Fig. 6).

Several experiments showed that clenbuterol protects control mice via β_2 -ARs. We first determined whether the β -AR antagonist propranolol prevented protection by clenbuterol. C57BL/6 mice were thus given propranolol (14 mg/l) for 48 h and clenbuterol (2.6 mg/l) plus propranolol (14 mg/l) for 17 h before injection of 15 μ g Jo2. They had the same death rate (Fig. 7A) and degree of liver apoptosis as untreated C57BL/6 mice (Fig. 4, G and H vs. C and D; Fig. 3A). We then examined whether other β_2 -AR agonists protected control mice against Jo2 MAb. The mice were therefore given 1.4 and 2.1 mg/l salbutamol for 17 h before Jo2 MAb injection. The agonist caused a dose-dependent delay in the lethal effect of the Jo2 MAb (Fig. 7B) and slightly but significantly reduced liver cell apoptosis (Fig. 3A).

DISCUSSION

Changes in cAMP concentration or activation of the cAMP-signaling pathway modulate the sensitivity of many cell types to apoptosis (1, 3, 7, 14, 18, 24, 26, 28,

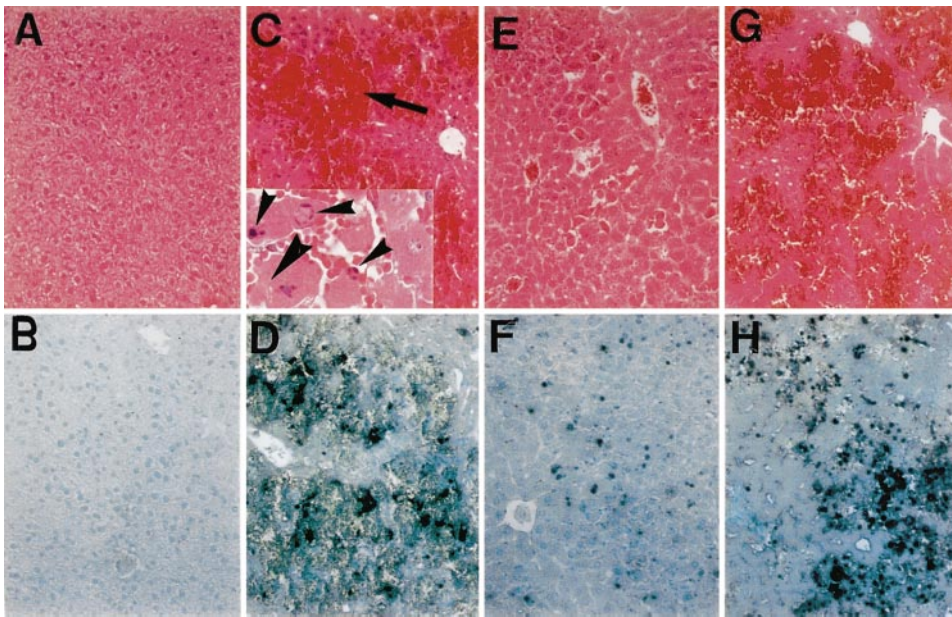


Fig. 4. Hematoxylin-eosin staining (A, C, E, G) and transferase UTP nick-end labeling (TUNEL) analysis (B, D, F, H) of liver sections from untreated C57BL/6 mice (A, B) and from C57BL/6 mice injected with Jo2 MAb that were untreated (C, D), given 1.3 mg/l clenbuterol (E, F), or given 2.6 mg/l clenbuterol plus 14 mg/l propranolol (G, H). Photographs were taken at $\times 100$ magnification. Intraparenchymatous hemorrhages, chromatin condensation, and cell surface blebbing are shown at $\times 400$ magnification in *inset of panel C* (arrow, small arrowheads, and large arrowhead, respectively). Results are representative of 2–3 sets of experiments performed on 3 animals each. Time point of histological slides in C, D, G, and H was ~ 3 h and in E and F was 5–6 h after Jo2 injection.

29, 37, 41) including the sensitivity of rat hepatocytes in culture to bile acid-induced and Fas-mediated apoptosis (11, 44). The liver of normal mice in vivo is particularly sensitive to the anti-Fas antibodies Jo2: they cause massive liver apoptosis in these animals that is followed by death in only a few hours, a phenotype that resembles fulminant hepatitis (33).

We have described a line of transgenic mice, F28, that carries the human β_2 -AR gene under the control of its own promoter and expresses the transgene in several organs. These mice overexpress functional β_2 -ARs in the liver (Table 1) and have a slightly increased number of β_2 -ARs in lung, heart, brain, and muscle (26). This study provides data suggesting that there is a higher production of cAMP in the liver of F28 mice compared with controls under normal physiological

conditions, although the hepatic cAMP levels in the two strains were similar under these conditions (Fig. 1A). Indeed, the livers of F28 mice accumulated about two times more cAMP than did those of control mice when they were treated *ex vivo* with the phosphodiesterase blocker IBMX to inhibit the metabolism of cAMP (Fig. 1B). We also showed that mice treated with the β_2 -AR agonist clenbuterol have a higher hepatic cyclase activity; this effect was considerably more marked in F28 mice than in C57BL/6 mice. The *in vivo* hepatic cAMP level in clenbuterol-treated F28 mice is significantly higher than in untreated F28 mice, despite the natural presence of phosphodiesterase activity (Fig. 1A). Also, *ex vivo* experiments done with IBMX not only confirmed that clenbuterol strongly increases cAMP production in the liver of F28 mice but also showed that the

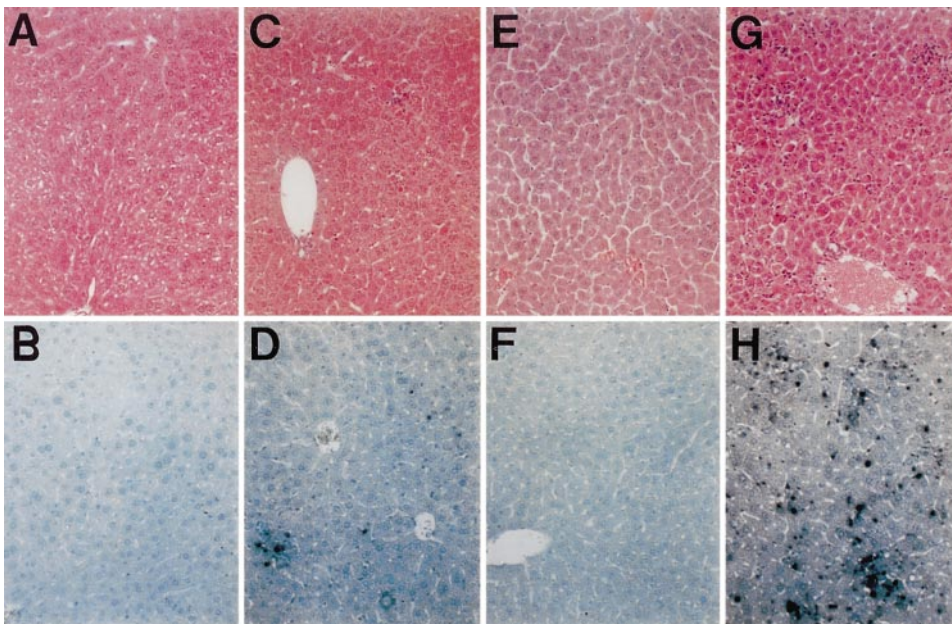


Fig. 5. Hematoxylin-eosin staining (A, C, E, G) and TUNEL analysis (B, D, F, H) of liver sections from untreated F28 mice (A, B) and from Jo2 MAb-injected F28 mice that were untreated (C, D), given 2.6 mg/l clenbuterol (E, F), or given 21 mg/l propranolol (G, H). Photographs were taken at $\times 100$ magnification. Results are representative of 2 individual sets of experiments, each performed on 3–4 mice. Time point of histological slides in C–H was 5–6 h after Jo2 injection.

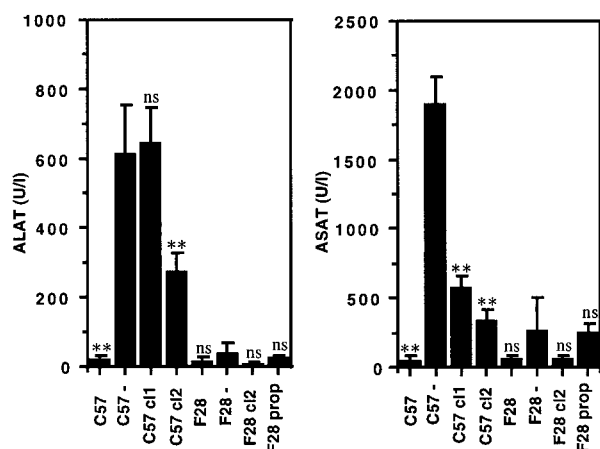


Fig. 6. Biochemical consequence of Jo2 injection in treated and untreated C57BL/6 and F28 mice. Serum activities of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined 3 h after injection of 15 μ g Jo2 in C57BL/6 mice treated with 1.3 or 2.6 mg/l clenbuterol (C57 cl1, C57 cl2), untreated C57BL/6 mice (C57-), F28 mice treated with 2.6 mg/l clenbuterol (F28 cl2) or with 21 mg/l propranolol (F28 prop), and untreated F28 mice (F28-). Noninjected mice served as controls (C57, F28). Data shown are means \pm SE of serum activities in 3–6 animals per group. Results were compared with C57- for C57BL/6 mice and with F28- for F28 mice. ** $P < 0.01$; ns $P > 0.05$.

agonist stimulates hepatic adenylyl cyclase activity in C57BL/6 mice, although to a lesser extent (Fig. 1B). Interestingly, ex vivo experiments strongly suggest that the hepatic cAMP level in F28 mice under normal physiological conditions is at least as high as the clenbuterol-induced hepatic cAMP level in C57BL/6 mice. The difference in the adenylyl cyclase activities observed in the liver tissues of the F28 and the control mice under nonstimulating conditions might simply be caused by stimulation of the β -AR systems by circulat-

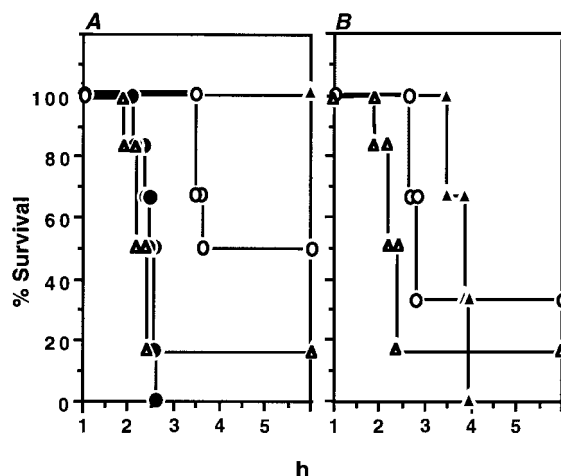


Fig. 7. Dose and β_2 -AR dependence of clenbuterol effect in C57BL/6 mice. Survival data of 6-wk-old C57BL/6 mice treated with 1.3 and 2.6 mg/l clenbuterol or 2.6 mg/l clenbuterol plus 14 mg/l propranolol (open circles, filled triangles, and filled circles, respectively; A) or 1.4 and 2.1 mg/l salbutamol (open circles and filled triangles, respectively; B) are shown. Results are compared with those for untreated mice (open triangles in A and B). Results are for 2–4 sets of experiments each with at least 3 mice per treatment. Data are expressed as percentages of mice that survived.

ing catecholamines. However, liver particulate fractions from F28 mice also had higher basal adenylyl cyclase activities than did those from control mice (Table 1). This suggests that an increase of spontaneously active receptors might contribute to the difference between the strains. Indeed, it was shown earlier that the overexpression of β_2 -ARs in mouse heart results in a higher basal adenylyl cyclase activity caused by more spontaneously active receptors (30). The higher maximum G protein-inducible adenylyl cyclase activity in F28 mouse liver particulate fractions (Table 1) suggests that a change in the amounts of the G proteins might also be involved.

Because the above findings indicate that a change in the abundance of functional hepatic β_2 -ARs and/or the administration of β -AR ligands may lead to a change in the adenylyl cyclase activity and consequently the production of intracellular cAMP, the major concern of the studies described here is to determine whether these also modulate the sensitivity of the mouse to Jo2 MAb in vivo.

It is shown that F28 mice are naturally protected against Jo2. There is no great difference in the amounts of Fas in F28 and C57BL/6 mice, but the F28 mice survive an intravenous injection of Jo2 and have only few apoptotic foci in their livers (Figs. 2 and 5). The protection of F28 mice against liver apoptosis is also partially inhibited by the potent β -AR antagonist propranolol (Figs. 3 and 5). This indicates that the overexpression of β_2 -ARs in the liver probably plays an important part in the natural resistance of F28 mice.

We have also demonstrated that the Jo2-induced hepatic apoptosis and lethality can be inhibited in vivo by giving β_2 -adrenergic agonists. F28 mice treated with the β_2 -AR-specific agonist clenbuterol have greater natural resistance to anti-Fas MAb than do untreated F28 mice (Fig. 5). Remarkably, although there are fewer hepatic β_2 -ARs in control mice (Table 1), clenbuterol also protects them from the lethal effect of the anti-Fas MAb (Fig. 2) and considerably reduces liver apoptosis (Figs. 3 and 4) and the biochemical sequels of liver destruction (Fig. 6) in a dose-dependent fashion (Fig. 7A). Several experiments confirmed that the protective effect of clenbuterol in control mice is caused by activation of β_2 -ARs: the β_2 -AR antagonist propranolol blocks the action of the β_2 -AR agonist clenbuterol (Figs. 3, 4, and 7A), whereas another β_2 -AR agonist, salbutamol, causes a dose-dependent delay in the lethal effect of the Jo2 MAb (Fig. 7B) and slightly reduces liver cell apoptosis (Fig. 3).

We do not yet know the mechanisms underlying the natural protection of F28 mice and the resistance conferred by the β -AR agonists. Activation of the cAMP-dependent protein kinase with cAMP or cAMP analogs inhibits the apoptosis of rat hepatocytes induced by polyclonal rabbit anti-murine Fas antibodies (M-20) (11). Data provided here strongly support the idea that activation of the cAMP-signaling pathways, modulation of hepatic adenylyl cyclase activity, and cAMP production are probably involved in altering the sensitivity of the liver to Jo2-mediated apoptosis in un-

treated F28 mice and in clenbuterol-treated mice. We have obtained evidence that clenbuterol activates the hepatic β_2 -AR system and that this activation can lead to increased hepatic cAMP production, which is twofold in normal mice and fivefold in F28 mice. Finally, cAMP production in untreated F28 mice is at least as high as in clenbuterol-treated normal mice. This might explain why even untreated F28 mice are that well protected against Jo2. This does not exclude the possibility that other regulatory mechanisms might be involved in the protection against Jo2 and even that there are differences between the regulatory mechanisms modulated by the hepatic β_2 -AR overexpression in the F28 mice and those that are triggered by clenbuterol. Stimulation of protein kinase A and regulation of protein phosphorylation are perhaps involved in the protective action of cAMP in hepatocytes (11, 44). The phosphorylation and dephosphorylation mechanisms regulating the Fas/Fas-L pathway are, however, very complex (17), and it is not known yet precisely which proteins are involved.

The regression of Jo2-induced mortality parallels the decrease in liver apoptosis mediated by β_2 -AR overexpression and β_2 -AR agonists (Figs. 2B and 7A vs. Fig. 3). The β_2 -AR agonist salbutamol has a smaller protective effect than does clenbuterol; it inhibits liver apoptosis less well and only delays the death of the mouse. The lower efficacy of this agonist is probably caused by differences in agonist potency and/or systemic bioavailability. This strongly suggests that the mouse is rescued from Jo2-induced death by reduction of liver apoptosis. A line of transgenic mice overexpressing Bcl-2 is protected against Jo2 MAb-induced liver apoptosis but not lethality, indicating that the stimulation of Fas on other target organs or cells could contribute to the lethal effect of Jo2 (35). Changing the β_2 -AR activity in other tissues might thus also be involved in the inhibition of the lethal effects of Jo2 MAb.

The expression of β_2 -ARs increases during liver regeneration after partial hepatectomy and in hepatocarcinoma (4, 15), whereas desensitization (which involves functional uncoupling, sequestration, and downregulation of the β -ARs, with a less active cAMP-signaling pathway and lower cAMP-dependent protein kinase activation of hepatic β_2 -ARs) is induced by chronic ethanol consumption (8), which can ultimately lead to cirrhosis. As we have shown that increased hepatic β_2 -AR activity diminishes the sensitivity of liver toward anti-Fas MAb-induced apoptosis and that a decreased hepatic β -AR activity makes hepatic cells more sensitive to anti-Fas MAb-induced apoptosis, it is possible that the regulation of the hepatic β_2 -AR/adenylyl cyclase system might also be implicated in the abnormal inhibition or stimulation of hepatocyte apoptosis.

β_2 - and β_1 -ARs are present in most tissues and on the cells of the immune system, and cAMP seems to be involved in regulating apoptosis in several types of cells (1, 3, 7, 11, 14, 18, 24, 26, 28, 29, 37, 41, 44). Hence, the regulation of β -AR expression might be important for regulating apoptosis in other tissues, and dysregula-

tion of β -AR expression might be implicated in the dysregulation of apoptosis that occurs in several major disorders. Several findings seem to support this. Desensitization of the cardiac β -AR system and apoptosis both occur in cardiac ischemia (5, 32), and β -AR ligands can modulate myocyte apoptosis (45, 46). The activity of the pulmonary β -AR system and the production of cytokines such as TNF- α are closely linked in chronic airway inflammation (see, e.g., Refs. 9, 12, 42). The abundance of the subsets of cells from the immune system changes considerably in immunologic responses, inflammation, and stress (see, e.g., Ref. 31), and this reorganization also involves changes in the expression of β -ARs on cells (21, 27) as well as the apoptotic regulation of cell survival (e.g., Ref. 43), which can be modulated by activation of the cAMP-dependent signaling pathway (see, e.g., Refs. 1, 18, 26).

We have shown here that mice can be protected in vivo from Jo2-induced massive liver apoptosis and death by increasing the hepatic β_2 -AR activity, either by increasing the number of β_2 -ARs or by stimulating the hepatic β_2 -AR-mediated signaling with synthetic agonists. This suggests new approaches to the treatment of diseases involving abnormal apoptosis in liver and perhaps, more generally, in other tissues or cells.

The authors thank Prof. A. D. Strosberg and Prof. A. Kahn for helpful discussions, Dr. B. Cheriou for serum analysis, and Dr. O. Parkes for editing the English text.

This work was supported by grants from the Association de Recherche contre le Cancer (ARC) and the Ligue Nationale contre le Cancer (LNC).

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Received 13 March 1998; accepted in final form 9 November 1998.

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