LIVER

Opposing effects of oestradiol and progesterone on intracellular pathways and activation processes in the oxidative stress induced activation of cultured rat hepatic stellate cells

T Itagaki, I Shimizu, X Cheng, Y Yuan, A Oshio, K Tamaki, H Fukuno, H Honda, Y Okamura, S Ito

Background: Oxidative stress, including the generation of reactive oxygen species (ROS), is involved in hepatofibrogenesis. The authors' previous studies have shown that oestradiol suppresses hepatic fibrosis in animal models and attenuates the activation of cultured rat hepatic stellate cells (HSCs), which possess oestrogen receptor subtype β and are also activated by ROS.

Aims: To define the mechanisms by which female sex hormones play an antifibrogenic role in activated HSCs, the effects of oestradiol and progesterone on ROS generation processes and intracellular pathways, leading to the activation of HSCs undergoing oxidative stress, was examined.

Methods: HSCs, isolated from rats, were cultured for 7 days with oestradiol or progesterone for 24 hours as pretreatment, and oxidative stress was then induced by exposure to low doses of hydrogen peroxide for another 24 hours.

Results: Oestradiol inhibited ROS generation and antioxidant enzyme loss via the suppression of NADH/NADPH oxidase activity, and attenuated hydrogen peroxide induced transforming growth factor-β1 (TGF-β1) expression, HSC proliferation and transformation, and the activation of mitogen activated protein kinase (MAPK) pathways and transcription factors. Progesterone exerted a stimulatory effect through the progesterone receptor on the induction of ROS generation processes and intracellular pathways, resulting in TGF-β1 expression and HSC activation, and fibrogenic effects were inhibited by oestradiol.

Conclusion: These findings show for the first time that oestradiol inhibits the activation of transcription factors by suppressing ROS generation processes and the MAPK pathways, and inactives the downstream transcription processes involved in TGF-β1 expression and HSC activation, whereas progesterone acts in opposition to the favourable effects of oestradiol and its effects are blocked by oestradiol.

Parenchymal cell membrane damage could produce reactive oxygen species (ROS) derived from lipid peroxidative processes, which represent the general feature of sustained inflammatory response and liver injury, and play a causative role in hepatic fibrosis development. Cells are well equipped to neutralise the effects of ROS, using a series of antioxidant protective systems, including enzymatic defence molecules such as superoxide dismutase (SOD), glutathione peroxidase, and catalase. Oestradiol is a potent endogenous antioxidant, which reduces lipid peroxidation and increases levels of SOD and glutathione peroxidase activity in the liver of a hepatic fibrosis model, and inhibits ROS generation in cultured rat hepatocytes in a state of prooxidant induced oxidative stress.2,3

We have already reported that oestradiol treatment results in the dose dependent suppression of hepatic fibrosis, accompanied by reduced collagen production in the liver of rat models of hepatic fibrosis, and attenuated iron induced lipid peroxidation in rat liver mitochondria.4 Hepatitis C virus infections are recognised as a major causative factor in the development of hepatic fibrosis to cirrhosis. Epidemiological studies support the view that cirrhosis development is more common in men than women with the exception of classical autoimmune diseases. Using multivariate analysis with classical liver enzymes in a protective role against the development of hepatic fibrosis.5

In the injured liver, hepatic stellate cells (HSCs) in the space of Disse are regarded as the primary target cells for inflammatory stimuli, and undergo proliferation and transformation into α smooth muscle actin (αSMA) positive myofibroblast-like cells. These HSCs are activated cells and are responsible for much of the collagen synthesis observed during hepatic fibrosis development to cirrhosis, although independent of age at the time of hepatitis C virus infection and of alcohol consumption, and that hepatic fibrosis begins to progress at 50 years of age, irrespective of the duration of virus infection.6 These findings implicate female sex hormones in a protective role against the development of hepatic fibrosis.

Abbreviations: αSMA, α smooth muscle actin; AP-1, activator protein-1; BrdU, bromodeoxyuridine; CuZn-SOD, zinc dependent SOD; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal regulated kinase; PBS, fetal bovine serum; H 2DCF-DA, 2,7’-dichlorofluorescein diacetate; HSC, hepatic stellate cell; JNK, c-Jun N-terminal kinase/stress activated protein kinase; MAPK, mitogen activated protein kinase; MDA, malondialdehyde; NF-kB, nuclear factor-κB; p38 MAPK; phosphatidylcholine; PBS, phosphate buffered saline; PDGF, platelet derived growth factor; p-ERK, phosphorylated ERK; p-JNK, phosphorylated JNK; RT-PCR, reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TGF-β1, transforming growth factor-β1.
Opposing effects of oestradiol and progesterone in HSCs

HSCs are activated by the generation of ROS with Fe²⁺/ascorbate and by malondialdehyde (MDA) and by 4-hydroxynonenal, end products of lipid peroxidation. In studies with cultured rat HSCs we have also shown that HSCs possess functional oestrogen receptor β, but not oestrogen receptor α of oestrogen receptor subtypes, which respond directly to oestradiol, and that oestradiol suppresses HSC activation with decreased proliferation and collagen production. However, there is little information about the possible role of progesterone, another female sex steroid, in hepatic fibrosis. Although in vitro studies showed that progesterone inhibited arterial smooth muscle cell proliferation, systemic hormone replacement therapy with oestrogens and progestins increases the dermal content of collagen. In contrast, progesterone combined with oestradiol attenuates the type I collagen synthesis and fibroblast proliferation of the anterior cruciate ligament in a dose dependent manner. This discrepancy might result from differences in the sex steroid compounds, the dosage combinations, and the experimental procedures employed. It remains to be elucidated whether progesterone can modulate HSC activation.

A primary source of ROS production in non-phagocytic cells such as HSCs is NADH/NADPH oxidase. Compared with the other ROS species, hydrogen peroxide is more stable and membrane permeable leading to the hypothesis that it acts as a second messenger to regulate signaling events including mitogen activated protein kinase (MAPK) activation. The MAPK family includes three major subgroups, extracellular signal regulated kinase (ERK), p38 MAPK (p38), and c-Jun N-terminal kinase/stress activated protein kinase (JNK). ERK and JNK lie upstream of activator protein-1 (AP-1). JNK and p38 activation are more important in stress responses such as inflammation, which can also activate nuclear factor κB (NF-κB). AP-1 and NF-κB are key transcription factors that induce multiple genes involved in inflammation and fibrogenesis, including cytokines and growth factors such as platelet derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1). TGF-β1 is a major fibrogenic cytokine, acting as a paracrine and autocrine (from HSCs) mediator, which triggers and activates the proliferation and transformation of HSCs in vivo.

To define the mechanisms by which female sex hormones might play an antifibrogenic role in activated HSCs, the effects of oestradiol and progesterone on NADH/NADPH oxidase activity, ROS generation, lipid peroxidation, and levels of SOD, catalase and glutathione peroxidase activity, as well as the activation of MAPK pathways and transformation factors of AP-1 and NF-κB, and TGF-β1 expression were examined in cultured rat HSCs undergoing oxidative stress.

**Materials and Methods**

**Isolation and culture of rat HSCs**

HSCs were isolated from the livers of male and female Wistar rats and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) on uncoated 35 mm diameter plastic dishes, as described previously. On day six of culture, the culture medium was removed, and cells were incubated in serum free DMEM with and without 17β-oestradiol (Sigma, St Louis, MO, USA) or progesterone (Wako, Osaka, Japan) for 24 hours. Oxidative stress was then induced by incubation with hydrogen peroxide for an additional 24 hours. In some experiments, cells were treated with α,β-D-glucosidase (Tocris Cookson, Ballwin, MO, USA), a high affinity oestrogen receptor antagonist, the progesterone receptor antagonist RU486 (Wako), or diphenylethionium (DPI, Sigma), a specific inhibitor of NADPH oxidases, in the presence and absence of oestradiol or progesterone for 24 hours. Steroid sex hormones and receptor antagonists were initially prepared as an ethanolic stock solution (10⁻⁷ mol/l) and then diluted with culture medium to the appropriate working solution concentration.

**Measurement of NADH and NADPH oxidase activities**

HSCs were washed five times, scraped in ice cold phosphate buffered saline (PBS), homogenised, and added to a cuvette containing lucigenin (500 μmol/l) as the electron acceptor and either NADH (100 μmol/l) or NADPH (100 μmol/l) as the substrate in 50 mmol/l phosphate buffer (pH 7.0). NADH or NADPH oxidase activity was measured by a luminescence assay, as previously reported. All measurements were expressed as nanomoles of substrate/minute per milligram of cellular protein. Protein concentrations were determined by the Lowry method.

**Measurement of intracellular ROS and lipid peroxidation**

Intracellular ROS was monitored using the 2′,7′-dichlorofluorescein diacetate (H₂DCF-DA) assay, as previously reported. H₂DCF-DA, dissolved in ethanol, was added at a final concentration of 10 μmol/l. 2′,7′-Dichlorofluorescein fluorescence was measured using a flow cytometer (EPICS XL, Coulter, Hialeah, FL, USA) with excitation at 488 nm and emission at 525 nm.

Lipid peroxidation in the cells was determined by measuring MDA levels, using the thiobarbiturate method, as described previously. Antioxidant enzyme assays

Protein levels of a predominantly cytosolic copper, zinc dependent SOD (CuZn-SOD) were detected using an enzyme linked immunosorbent assay (ELISA) system kit (Amersham, Little Chalfont, UK), and glutathione peroxidase activities were determined using a Cellular Glutathione Peroxidase Assay kit (Calbiochem, San Diego, CA, USA), as described previously. The results are expressed as nanograms of immunoreactive protein levels for CuZn-SOD and the units for glutathione peroxidase per milligram of cellular protein. Catalase proteins were detected immunologically as described below.

**Western blot analysis**

Cell lysates were obtained with a sodium dodecyl sulfate (SDS) loading buffer as described previously. Ten micrograms of cell associated proteins were electrophoresed on 12% SDS-polyacrylamide gels, and the proteins were then transferred onto Hybond-ECL membranes (Amersham, Arlington Heights, IL, USA). Membranes were incubated in...
blocking buffer containing antibodies against catalase (Sigma), αSMA (DAKO, Glostrup, Denmark), IκB-α (inhibitory subunit of NF-κB; New England Biolabs, Beverly, MA, USA) and intracellular signaling proteins of ERK (Zymed, San Francisco, CA, USA), phosphorylated ERK (p-ERK) (Santa Cruz), p38 (Santa Cruz), phosphorylated p38 (p-p38) (Santa Cruz), JNK (Santa Cruz), and phosphoformylated JNK (p-JNK) (Santa Cruz). A rabbit antibody against β-actin (Biomedical Technologies, Stoughton, MA, USA) was used to control protein loading. All antibodies were used at a dilution of 1:1000. Immunoreactive bands were visualised with an ECL western blotting detection system (chemiluminescence) kit (Amersham) as described previously, scanned with Gel Doc 2000 (Bio-Rad, Hercules, CA, USA) and analysed using Quantity One software (Bio-Rad).

Cell proliferation
Using a Biotrak cell proliferation ELISA system (Amersham), DNA synthesis was measured by incubating cells in 96-well plates with 10 μmol/l of the pyrimidine analogue, bromodeoxyuridine (BrdU) for 24 hours. After removing the culture medium, the cells were fixed and the incorporated BrdU was detected according to the manufacturer’s instructions, then compared with the effects of a positive control, PDGF-BB (5 ng/ml, Calbiochem).

ELISA analysis of TGF-β1 protein
To determine TGF-β1 protein, cells were rinsed with cold PBS and removed by scraping as described previously. Rat TGF-β1 in the cells was measured using an ELISA kit (R&D Systems) according to the manufacturer’s instructions.

Reverse transcription-polymerase chain reaction
Cells were collected using ISOGEN (Nippon gene, Tokyo, Japan) and total RNA was prepared according to the manufacture’s instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a one-step RNA PCR kit (AMV) (Takara, Kyoto, Japan). Briefly, 1 μg of total RNA for each sample was added to the PCR reaction. For the gene expression of the progesterone receptor, after reverse transcription (30 minutes at 50 ºC; 2 minutes at 94 ºC), 30 cycles of PCR were performed for 30 seconds at 94 ºC, 45 seconds at 61 ºC, and 1 minute at 72 ºC, and the final elongation time was 7 minutes at 72 ºC. The set of primers was described previously. The resultant fragment of the progesterone receptor was 320 bp in size. Rat uterus was used
as a positive control for the progesterone receptor, and yeast transfer RNA was used as a negative control.

For TGF-β1 gene expression, real-time PCR was performed using a SYBR Green PCR Core Reagents kit (Biosystems, Warrington, UK) according to the manufacturer's protocol. The primer sequences were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA): 5’-TGGCTACTCTACTGTTCC-3’ for the forward primer and 5’-GGTGGTGAGCCCTTCC-3’ for the reverse primer. The primers for 18S rRNA were purchased form a commercial vendor. Quantitative PCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol as follows: 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles with denaturation at 95°C for 15 seconds, and annealing at 60°C for 1 minute. The specificity of the produced amplification products was confirmed by examination of the dissociation reaction plots.7 8 A distinct single peak indicated that a single DNA sequence was amplified during PCR.

Electrophoretic mobility shift assay
Cellular extract proteins were prepared as described elsewhere.7 For the electrophoretic mobility shift assay (EMSA), an oligonucleotide corresponding to the DNA binding consensus site for AP-1 or NF-κB was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase using an AP-1 family (T-cell activation) Gelshift kit (Geneka Biotechnology, Quebec, Canada) or an NF-κB family (T-cell activation) Gelshift kit (Geneka Biotechnology) as previously reported.9 The bandshift was visualised by autoradiography. Competition was performed by adding specific unlabeled double stranded oligonucleotide to the reaction mixture in a 20- or 100-fold molar excess.

Statistical analysis
Data are presented as the mean (standard deviation), unless otherwise indicated. Means were compared between two groups using Wilcoxon’s signed rank test and the Mann-Whitney U test. All p values are two tailed. A p value of less than 0.05 was considered to be statistically significant.

RESULTS
Protein and gene expressions of progesterone receptor in cultured rat HSCs with and without hydrogen peroxide exposure
We performed western blot analysis of extracts from the rat uterus using the antiprogesterone receptor antibody (C-20). When lysates of seven day cultured HSCs (fig 1A) obtained from male and female rats were analysed by western blotting in the same manner, we found 94 kD and 114 kD bands for the progesterone receptor. 29 The progesterone receptor in the same manner, we found 94 kD and 114 kD bands for the progesterone receptor in cultured rat HSCs with and without hydrogen peroxide exposure.

Protein and gene expressions of progesterone receptor in cultured rat HSCs with and without hydrogen peroxide exposure

RESULTS
Protein and gene expressions of progesterone receptor in cultured rat HSCs with and without hydrogen peroxide exposure

Effects of oestradiol and progesterone on the activation of MAPK pathways, transcription factors, and TGF-β1 expression in cultured rat HSCs with and without hydrogen peroxide exposure

On culture day seven, exposure to low doses of hydrogen peroxide (10^{-7}\text{ to }10^{-5}\text{ mol/l}) for 24 hours in HSCs preincubated in serum-free DMEM for 24 hours was then exposed to 10^{-5}\text{ mol/l} hydrogen peroxide for another 24 hours. Following exposure to hydrogen peroxide, α-SMA expression (A), DNA synthesis (B), and intracellular levels of TGF-β1 mRNA and protein (C) were increased after hydrogen peroxide exposure. DNA synthesis in the presence of PDGF-BB (5 ng/ml) for 1 hour, as a positive control, was 142% (SD 10%). Results of densitometric analysis are presented as the mean percentages of β-actin signal intensity of for α-SMA expression (B). The levels of TGF-β1 gene expression were quantitatively analysed by real-time PCR, and the results are expressed in arbitrary units (C). Values are means (SD) for six dishes. *p<0.05 compared with cultures before hydrogen peroxide exposure.

Figure 3: Stimulation of proliferation and α-SMA expression and intracellular levels of TGF-β1 mRNA and protein after exposure to hydrogen peroxide in cultured rat HSCs.
Results

Effects of oestradiol and progesterone on NADH/NADPH oxidase activity, ROS generation, lipid peroxidation, and antioxidant enzyme levels in cultured rat HSCs without hydrogen peroxide exposure

The effect of oestradiol and progesterone in cultured HSCs on the activity of NADH/NADPH oxidase was evaluated by measuring the photon emission and the initial rate of NADH or NADPH oxidase activity. On culture day seven, HSCs, preincubated in serum-free DMEM for 24 hours, were then incubated for another 24 hours in the presence and absence of oestradiol (10^{-7}–10^{-5} mol/l) without hydrogen peroxide exposure. The activities of both NADH and NADPH oxidases decreased in the presence of oestradiol in a dose dependent manner, compared with cultures in the absence of oestradiol without hydrogen peroxide exposure (none) (table 2). In addition, oestradiol treatment led to the dose dependent inhibition of intracellular ROS and MDA generation and the loss of antioxidant enzyme levels of CuZn-SOD, glutathione peroxidase, and catalase expression (table 2). The inhibitory effects of oestradiol were blocked by 10^{-7} mol/l ICI 182,780. These findings suggest that oestradiol is able to inhibit ROS generation, lipid peroxidation, and antioxidant enzyme loss via the suppression of NADH/NADPH oxidase activity through the oestrogen receptor. However, it cannot be deduced from the data whether oestrogen binding of the oestrogen receptor may affect the expression of antioxidant enzymes at the transcriptional level. In contrast, treatment with progesterone (10^{-7}–10^{-5} mol/l) for 24 hours induced a dose dependent enhancement in ROS generation, lipid peroxidation, and antioxidant enzyme loss via NADH/NADPH oxidase activation (table 2). The stimulatory effects of progesterone were blocked by 10^{-6} mol/l RU486, suggesting that progesterone exerted its stimulatory effects through the progesterone receptor.

Effects of oestradiol on progesterone induced NADH/NADPH oxidase activity, lipid peroxidation, proliferation, αSMA expression, and TGF-β1 expression in cultured rat HSCs with hydrogen peroxide exposure

Hydrogen peroxide exposure for 24 hours resulted in a further enhancement in NADH/NADPH oxidase activity, lipid peroxidation, and antioxidant enzyme loss in cultured HSCs compared with cultures without hydrogen peroxide exposure. In the oxidative stress induced activation of cultured HSCs, progesterone induced a further increase in NADH/NADPH oxidase activity and intracellular ROS and MDA generation as well as proliferation, αSMA, and intracellular levels of TGF-β1 mRNA and protein (table 3) and the activation of oestradiol (10^{-9}–10^{-7} mol/l) (fig 4 and table 1). The inhibitory effects of oestradiol at a dose of 10^{-7} mol/l on the activation of MAPK pathways and transcription factors, the degradation of IκBα, and the stimulation of proliferation, αSMA expression, and TGF-β1 expression were blocked by the specific ER antagonist ICI 182,780, in a dose dependent manner (data not shown), which was complete at 10^{-7} mol/l ICI 182,780 (fig 4 and table 1).

In contrast to oestradiol, progesterone pretreatment for 24 hours resulted in the further activation of MAPK pathways and transcription factors and further stimulation of proliferation, αSMA expression and TGF-β1 expression in the hydrogen peroxide induced activation of cultured HSCs. The effect of progesterone (10^{-7}–10^{-5} mol/l) was dose dependent and was blocked by the progesterone receptor antagonist RU486 at a dose of 10^{-6} mol/l (fig 4 and table 1). Treatment with ICI 182,780 or RU486 alone had no effect on any parameters examined herein (data not shown).

Figure 4 Effects of oestradiol and progesterone on the activation of MAPK pathways and transcription factors of AP-1 and NF-κB in cultured rat HSCs with and without hydrogen peroxide exposure. On culture day seven, oxidative stress was induced in HSCs by exposure to 10^{-5} mol/l hydrogen peroxide (oxidative stress) for 24 hours in pretreatment in serum-free DMEM with and without oestradiol (10^{-7}–10^{-5} mol/l) or progesterone (10^{-7}–10^{-5} mol/l) in the presence and absence of 10^{-6} mol/l ICI 182,780 (ICI) or 10^{-6} mol/l RU486 (RU) for 24 hours. After exposure to hydrogen peroxide, activation of the MAPK pathways of ERK, p38, and JNK (A) was evaluated at 30 minutes, activation of transcription factors of AP-1 and NF-κB (B) with the degradation of IκBα (C) at 1 hour. The micrographs represent typical results of three independent experiments.

AP-1 and NF-κB and the degradation of inhibitory protein of NF-κB, IκBα were observed, as shown in figures 2B and C. The specificity of AP-1 or NF-κB DNA binding induced by hydrogen peroxide was confirmed in competition experiments. Incubation with excess mutant oligonucleotide had no effect on AP-1 or NF-κB binding (data not shown). The activation of AP-1 and NF-κB as determined by EMSA and the degradation of IκBα, as detected by western blot analysis were readily detected after 15 minutes and peaked after 1 hour (figs 2B and C). The DNA synthesis and αSMA expressions peaked after 1 hour and 24 hours, respectively (fig 3A) as did the intracellular levels of TGF-β1 mRNA and protein (figs 3B and C). Subsequent studies used an incubation time of 30 minutes to activate MAPK pathways, 1 hour for the activation of AP-1 and NF-κB with degradation of IκBα, DNA synthesis, and TGF-β1 mRNA level, and 24 hours for αSMA expression and TGF-β1 protein level, after hydrogen peroxide exposure.

The hydrogen peroxide induced activation of MAPK pathways and transcription factors of AP-1 and NF-κB, the degradation of IκBα, and stimulation of DNA synthesis, αSMA expression, and intracellular expression of TGF-β1 in the cultures were inhibited in a dose dependent manner by
MAPK pathways and transcription factors in a dose dependent manner (data not shown). The stimulatory effects of progesterone at a dose of $10^{-7}$ mol/l were inhibited by simultaneous coinubcation with oestradiol ($10^{-5}$–$10^{-7}$ mol/l) in a dose dependent manner. Treatment with $10^{-6}$ mol/l ICI 182,780 led to a complete block of oestradiol mediated responses to progesterone exposure in cultured HSCs in a state of oxidative stress (table 3).

To assess the central or main role of the NADH/NADPH oxidase, a separate series of competition experiments was conducted with the NADH/NADPH oxidase inhibitor DPI ($10^{-7}$ mol/l) in cultured HSCs (table 3). DPI treatment in HSCs cultured with hydrogen peroxide exposure induced a decrease in NADH/NADPH oxidase activity and the intracellular generation of ROS and MDA as well as the proliferation, αSMA and intracellular levels of TGF-β1 mRNA and protein.

The stimulatory effects of progesterone were blocked by DPI, and the concomitant treatment of oestradiol with DPI resulted in further inhibition in the parameters examined herein (table 3).

**DISCUSSION**

This study indicates for the first time that there is a competing regulation between the actions of oestradiol and progesterone on oxidative stress induced ROS generation mainly originating from NADH/NADPH oxidase, the activation of MAPK pathways and transcription factors of AP-1 and NF-κB, TGF-β1 expression, proliferation, and transformation in cultured rat HSCs. Oestradiol was found to inhibit ROS generation, lipid peroxidation, and antioxidant enzyme loss via the suppression of NADH/NADPH oxidase activity and to attenuate the hydrogen peroxide induced activation of ERK.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BMU incorporation (% of control)</th>
<th>αSMA mRNA (relative protein level)</th>
<th>TGF-β1 mRNA (arbitrary unit)</th>
<th>TGF-β1 protein (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 (7)*</td>
<td>55 (13)*</td>
<td>0.58 (0.08)*</td>
<td>15.1 (2.7)*</td>
</tr>
<tr>
<td>+ Oxidative stress only</td>
<td>147 (9)</td>
<td>116 (20)</td>
<td>1.00 (0.21)</td>
<td>39.8 (6.1)*</td>
</tr>
<tr>
<td>+ Progestosterone $10^{-7}$ mol/l</td>
<td>184 (11)*</td>
<td>146 (25)*</td>
<td>1.47 (0.14)</td>
<td>44.5 (8.5)*</td>
</tr>
<tr>
<td>+ Progestosterone $10^{-6}$ mol/l</td>
<td>205 (15)*</td>
<td>168 (28)*</td>
<td>1.02 (0.18)</td>
<td>29.2 (6.9)*</td>
</tr>
<tr>
<td>+ Oxidative stress + RU</td>
<td>151 (9)</td>
<td>118 (21)</td>
<td>0.70 (0.09)</td>
<td>21.9 (5.3)*</td>
</tr>
<tr>
<td>+ Oxidative stress + Oestradiol $10^{-6}$ mol/l</td>
<td>123 (9)*</td>
<td>96 (13)*</td>
<td>0.61 (0.08)*</td>
<td>17.4 (3.5)*</td>
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<tr>
<td>+ Oxidative stress + Oestradiol $10^{-7}$ mol/l</td>
<td>106 (8)*</td>
<td>75 (15)*</td>
<td>0.53 (0.07)*</td>
<td>13.3 (3.1)*</td>
</tr>
<tr>
<td>+ Oxidative stress + Oestradiol $10^{-8}$ mol/l</td>
<td>90 (7)*</td>
<td>54 (10)*</td>
<td>0.58 (0.08)*</td>
<td>7.8 (1.0)*</td>
</tr>
<tr>
<td>+ Oxidative stress + DPI</td>
<td>146 (9)</td>
<td>113 (21)</td>
<td>0.95 (0.19)</td>
<td>28.5 (5.1)*</td>
</tr>
</tbody>
</table>

On culture day seven, oxidative stress was induced in HSCs by exposure to $10^{-6}$ mol/l hydrogen peroxide (oxidative stress) for 24 hours in pretreatment with serum-free DMEM with and without oestradiol ($10^{-5}$–$10^{-7}$ mol/l) or progesterone ($10^{-6}$–$10^{-8}$ mol/l) in the presence and absence of $10^{-6}$ mol/l RU486 (RU) or $10^{-6}$ mol/l ICI 182,780 (ICI) for 24 hours. After exposure to hydrogen peroxide, DNA synthesis and TGF-β1 mRNA levels were evaluated at 1 hour, and αSMA expression and TGF-β1 protein level at 24 hours. The level of DNA synthesis showed a dose dependent manner, was 140 (SD 9%). The results of densitometric analysis are presented as mean percentages of the signal intensity of β-actin for αSMA expression. The levels of TGF-β1 gene expression were quantitatively analysed by real-time PCR, and results are expressed in arbitrary units. Values are means (SD) for six dishes. *p<0.05 compared with cultures after hydrogen peroxide exposure (oxidative stress).

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NADH oxidase (mmol/min/mg protein)</th>
<th>NADPH oxidase (mmol/min/mg protein)</th>
<th>ROS channel number/mg protein</th>
<th>MDA (mmol/mg protein)</th>
<th>CuZn-SOD (ng/mg protein)</th>
<th>Glutathione peroxidase (U/mg protein)</th>
<th>Catalase (relative protein level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>29 (0.1)</td>
<td>8.1 (1.3)</td>
<td>7.5 (1.2)</td>
<td>0.46 (0.07)</td>
<td>3.2 (0.4)</td>
<td>2.0 (0.3)</td>
<td>65 (12)</td>
</tr>
<tr>
<td>+ Progestosterone $10^{-7}$ mol/l</td>
<td>37 (6.6)*</td>
<td>10.6 (2.1)*</td>
<td>9.3 (1.5)*</td>
<td>0.58 (0.09)*</td>
<td>2.7 (0.4)*</td>
<td>1.5 (0.3)*</td>
<td>53 (10)*</td>
</tr>
<tr>
<td>+ Progestosterone $10^{-1}$ mol/l</td>
<td>43.5 (9.4)</td>
<td>13.9 (2.9)</td>
<td>11.0 (2.2)*</td>
<td>0.68 (0.10)*</td>
<td>2.1 (0.5)*</td>
<td>1.1 (0.2)*</td>
<td>39 (10)*</td>
</tr>
<tr>
<td>+ Progestosterone $10^{-6}$ mol/l</td>
<td>30.6 (3.4)</td>
<td>8.6 (1.3)</td>
<td>7.8 (1.0)</td>
<td>0.48 (0.06)</td>
<td>3.2 (0.5)</td>
<td>2.0 (0.4)</td>
<td>65 (11)</td>
</tr>
<tr>
<td>+ RU</td>
<td>250 (13.3)*</td>
<td>7.5 (1.0)*</td>
<td>6.0 (1.0)*</td>
<td>0.33 (0.06)</td>
<td>4.5 (0.7)*</td>
<td>2.6 (0.4)*</td>
<td>82 (16)*</td>
</tr>
<tr>
<td>+ Oestradiol $10^{-6}$ mol/l</td>
<td>22.0 (3.2)*</td>
<td>5.8 (0.8)*</td>
<td>4.8 (0.7)*</td>
<td>0.30 (0.05)*</td>
<td>7.0 (1.3)*</td>
<td>4.0 (0.9)*</td>
<td>119 (22)*</td>
</tr>
<tr>
<td>+ Oestradiol $10^{-7}$ mol/l</td>
<td>16.9 (2.4)*</td>
<td>4.8 (0.6)*</td>
<td>3.8 (0.5)*</td>
<td>0.22 (0.04)*</td>
<td>8.1 (1.4)*</td>
<td>4.8 (1.1)*</td>
<td>140 (27)*</td>
</tr>
<tr>
<td>+ Oestradiol $10^{-8}$ mol/l</td>
<td>28.5 (4.1)*</td>
<td>7.9 (1.3)</td>
<td>7.4 (1.1)</td>
<td>0.46 (0.07)</td>
<td>3.4 (0.6)</td>
<td>2.0 (0.4)</td>
<td>64 (15)</td>
</tr>
</tbody>
</table>

On culture day seven, HSCs, preincubated in serum-free DMEM for 24 hours, were then incubated with and without oestradiol ($10^{-7}$–$10^{-8}$ mol/l) or progesterone ($10^{-7}$–$10^{-8}$ mol/l) in the presence and absence of $10^{-6}$ mol/l ICI 182,780 (ICI) or $10^{-6}$ mol/l RU486 (RU) for another 24 hours without hydrogen peroxide exposure. NADH/NADPH oxidase activities, the generation of ROS and MDA, and the levels of CuZn-SOD, glutathione peroxidase, and catalase expression were then compared with cultures in the absence of steroid sex hormones and receptor antagonists without hydrogen peroxide exposure (none). The densitometric analysis results of catalase expression are presented as mean percentages of β-actin signal intensity. Values are means (SD) for six dishes. *p<0.05 compared with cultures without any treatment (none).
indicating that hydrogen peroxide at a dose of 10^{-2} mol/l induced transcription factors AP-1 and NF-kB. The stress signals, followed by the stimulation of at least two well-defined transcription factors, TGF-β, ERK, and p38, and the degradation of IκBα, TGF-β1 expression, and HSC activation through the oestrogen receptor in a dose dependent manner. In contrast, HSCs were observed to possess the progesterone receptor using immunohistochemistry, western blotting, and RT-PCR. Progesterone treatment induced the dose dependent enhancement of ROS generation, lipid peroxidation, and antioxidant enzyme loss via NADH/NADPH oxidases, representing the most important ROS (channel 240, 0.05 compared with cultures after hydrogen peroxide exposure). The levels of TGF-β1 gene expression were quantitatively analysed by real-time PCR, and the results are expressed in arbitrary units. Values are means (SD) for six dishes. *p<0.05 compared with cultures after hydrogen peroxide exposure (oxidative stress). †p<0.05 compared with cultures treated with progesterone alone after hydrogen peroxide exposure.

| Table 3 Effects of oestradiol on progesterone induced NADH/NADPH oxidase activities, ROS generation, lipid peroxidation, proliferation, nSMA expression, and intracellular levels of TGF-β1 mRNA and protein in cultured rat HSCs with hydrogen peroxide exposure |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| NADH oxidase                   | NADPH oxidase | ROS channel    | MDA (mmol/    | BrDU (relative |
| (mmol/min/mg protein)          | (mmol/min/    | number/mg protein) | mg protein)  | (relative mRNA) |
|                                 | mg protein)  |                |                |                |
| None                            | 28.9 (5.5)†   | 8.1 (2.1)†     | 7.7 (1.1)†    | 0.52 (0.14)†   |
| + Oxidative stress only         | 78.3 (14.8)   | 27.2 (5.2)     | 20.9 (4.0)    | 1.44 (0.23)†   |
| + Oxidative stress              | 31.4 (5.4)†   | 9.0 (2.1)†     | 7.8 (1.1)†    | 0.55 (0.11)†   |
| + DPI 10^{-6} mol/l             | 111.1 (17.3)† | 35.1 (6.2)     | 29.5 (4.5)†   | 1.92 (0.32)†   |
| + Progestosterone 10^{-6} mol/l | 32.3 (5.6)†   | 9.4 (2.5)†     | 8.5 (1.2)†    | 0.58 (0.14)†   |
| + Oxidative stress              | 73.6 (13.2)†  | 22.4 (4.3)     | 19.0 (3.5)†   | 1.24 (0.22)†   |
| + Progestosterone 10^{-6} mol/l | 50.2 (13.3)†  | 14.5 (4.2)†    | 13.3 (2.8)†   | 0.81 (0.18)†   |
| + Estradiol 10^{-6} mol/l       | 110.5 (16.1)† | 35.7 (6.4)     | 28.4 (4.3)†   | 1.87 (0.29)†   |
| + Oxidative stress              | 33.8 (6.1)†   | 9.9 (2.5)†     | 8.3 (1.5)†    | 0.59 (0.15)†   |

On culture day seven, oxidative stress was induced in HSCs by exposure to 10^{-9} mol/l hydrogen peroxide (oxidative stress) for 24 hours in the pretreatment in serum-free DMEM with and without progesterone (10^{-6} mol/l) and/or oestradiol (10^{-6}–10^{-7} mol/l) in the presence and absence of 10^{-6} mol/l ICI 182,780 or 10^{-6} mol/l DPI for 24 hours. NADH/NADPH oxidase activities, the generation of ROS and MDA were then compared with cultures treated with progesterone alone after hydrogen peroxide exposure. DNA synthesis and TGF-β1 mRNA levels were evaluated at 1 hour, and nSMA expression and TGF-β1 protein level at 24 hours. DNA synthesis in the presence of PDGF-BB (5 ng/ml), as a positive control, was 148 (SD 9)%.

Oestradiol and its derivatives (2-hydroxyestradiol) are strong endogenous antioxidants that reduce lipid peroxide levels in the liver and serum.43 4 This study confirms that oestradiol mediated inactivation of HSCs with the suppressed oestrogen receptor in a dose dependent manner. In contrast, HSCs were observed to possess the progesterone receptor using immunohistochemistry, western blotting, and RT-PCR. Progesterone treatment induced the dose dependent enhancement of ROS generation, lipid peroxidation, and antioxidant enzyme loss via NADH/NADPH oxidases, representing the most important ROS (channel 240, 0.05 compared with cultures after hydrogen peroxide exposure). The levels of TGF-β1 gene expression were quantitatively analysed by real-time PCR, and the results are expressed in arbitrary units. Values are means (SD) for six dishes. *p<0.05 compared with cultures after hydrogen peroxide exposure (oxidative stress). †p<0.05 compared with cultures treated with progesterone alone after hydrogen peroxide exposure.
The stimulatory effects of progesterone were blocked by the progesterone receptor antagonist RU486. In cultured vascular smooth muscle cells, although several contradictory reports exist concerning the effect of progesterone on cell proliferation, this is the first report to address the fibrogenic response of progesterone through the progesterone receptor in HSCs to low levels of oxidative stress, and the inhibitory effect of oestradiol on progesterone induced HSC activation. This suggests that the resultant inhibition of HSC activation and hepatic fibrosis induced by the combinations of oestradiol and progesterone at physiological relevant concentrations may help explain the preponderance of men suffering from chronic liver disease.

In the TGF-β1 response to oestradiol, it was reported that systemic hormone replacement therapy with oestrogens and progesterone decreased human cutaneous wound healing, associated with the upregulation of TGF-β1. However, our previous study showed that catalase treatment resulted in decreased ROS generation and TGF-β1 levels in cultured HSCs. It has been proposed that ROS, particularly hydrogen peroxide, acts as a signalling mediator for TGF-β1. De Bleser et al reported that exogenous TGF-β1 increased the production of hydrogen peroxide by HSCs, whereas the addition of systemic hormone replacement therapy with oestrogens and progesterone at physiological relevant concentrations may help explain the preponderance of men suffering from chronic liver disease.

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Authors’ response

We have read with interest the letter by Pineton de Chambrun and colleagues. The letter reflected on our prospective study of JC viral loads in immunosuppressed patients with Crohn’s disease and in controls. We thank the colleagues from Lille for their interest in our work and for further exploring this difficult issue, which will be crucial for the further development of anti-integrin therapies to treat inflammatory disorders. In the five patients with human immunodeficiency virus (HIV) diagnosed with progressive multifocal leukoencephalopathy (PML), the authors could not detect JC viraemia in the months preceding the clinical diagnosis. This finding is in contrast with the case of PML reported in a patient treated with the anti-α4 integrin monoclonal antibody natalizumab.

However, the authors correctly state that the data available in the literature on the occurrence of JC viraemia before the development of symptomatic PML are inconsistent.

We would like to respond to some of the issues raised by the authors. First, the diagnosis of PML in the five patients described appears sound and adds to the value of exploring JC virus replication. However, in contrast to our prospective trial incorporating 351 patients and controls, this small retrospective study has inherent limitations. First, blood sampling was not planned ahead of time and varied substantially between patients. Stored serum was available at the time of diagnosis in only two of the five patients and both these two patients had positive JC viraemia. In the months before the clinical diagnosis of PML sampling was highly variable and this generates an important risk for sampling bias and for missing transient viraemia. Second, it is unknown when these PML cases occurred, how rapidly the samples were frozen and how many thawing cycles they were subjected to. Given the relatively low JC viral loads found in the positive patients (3.0 × 10^6 to 1.0 × 10^7 copies/ml) it is possible that in some of the samples the viral DNA degraded and JC viral loads fell below the limit of detection (1.1 × 10^6 copies/ml). Third, the state of immunosuppression induced by HIV-AIDS is clearly more profound and different from the medical immunosuppression associated with the use of steroids, azathioprine or anti-tumour necrosis factor (TNF) agents. The pathways by which JC virus travels to and reactivates in the brain may be similar for all types of immunosuppression, but there are no data to support that hypothesis. Therefore, we believe that specific studies in immunosuppressed patients with inflammatory bowel disease (IBD) are needed and that we cannot extrapolate data from patients with HIV-AIDS or haematological malignancies. Finally, even if JC viral loads in the cerebrospinal fluid (CSF) correlate better with PML, spinal taps are too invasive for regular screening. Furthermore, the appearance of JC virus in the CSF is probably a late phenomenon when irreversible encephalopathy has already started.

Optimising the long-term benefit to risk ratio is of paramount importance in the treatment of chronic immune disorders such as Crohn’s disease. The medical need for biological agents other than anti-TNF agents and the promising results with anti-integrin therapies in IBD, are inevitably being weighed against the limited but real risk of PML. We agree with the colleagues from Lille, that any attempt to develop a non-invasive screening strategy for early detection of JC virus replication relevant to the development of the devastating and irreversible damage PML causes in the brain, should be fostered.

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CORRECTIONS

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