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Iron overload and gene expression in HepG2 cells: analysis by differential display

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Abstract The aim of the present study was to evaluate the effect of iron overload on gene expression in HepG2 cells by differential display. Iron-treated cells showed a 50% decrease in apolipoprotein B100 (Apo B100) and a 2- and 3-fold increase in semaphorin cd100 and aldose reductase mRNA, respectively, with parallel variations in Apo B100 and aldose reductase proteins. These effects were time-dependent. Vitamin E prevented the increase in aldose reductase expression, but had no effect on Apo B100 and semaphorin cd100. Treatment with hydrogen peroxide and 4-hydroxy-2,3-nonenal increased only aldose reductase mRNA. These data suggest that iron can affect mRNA levels by lipid peroxidation-dependent and -independent pathways.

Key words: Differential display; Hepatocyte; Antioxidant; Apolipoprotein B; Semaphorin cd100; Aldose reductase

1. Introduction

Iron is essential for a series of cell activities, including cytochromes and oxidases, but an excess of intracellular iron may be toxic since it can produce highly reactive hydroxyl radicals from H_2O_2, and ferryl ions by complexing with oxygen [1], both of which can initiate lipid peroxidation. In turn, lipid peroxidation of cell membranes can generate organelle dysfunction and cell death (reviewed in [2]).

This potential toxicity requires the close regulation of iron metabolism, a result mainly obtained by controlling the expression of the genes directly involved in iron uptake and storage. The binding of iron regulatory proteins to the iron regulatory elements (IREs) of transferrin receptor (TfR) and ferritin mRNAs can control mRNA stability or the initiation of translation, and thus regulate gene expression at a post-transcriptional level (reviewed in [3]).

However, iron can also affect the gene transcription of proteins directly involved in its metabolism, as observed for ferritin in primary human hepatocytes [4] and Friend leukemia cells [5]. Moreover, increased intracellular iron levels decrease inducible nitric oxide synthase transcription in a macrophage cell line [6]. Iron overload also induces the expression of genes involved in the cellular response to oxidative stress, such as heme oxygenase 1 [7], glutathione peroxidase [8] and glyceraldehyde 3-phosphate dehydrogenase [9].

These iron effects may be mediated by modifications of transcription factors activity, since inhibition of NF-kB and increase in NF-IL6 activity due to iron chelation have recently been demonstrated [10,11]. The mechanisms by which iron affects gene transcription may include oxidative stress and lipid peroxidation. Oxidative stress activates NF-kB and activator protein-2 in rat liver [12]; furthermore, NF-kB activation in hepatocytes can be prevented by vitamin E [13].

The aim of this study was thus to evaluate the effect of iron overload on gene expression in an in vitro model of HepG2 cells, a well-differentiated cell line that maintains most hepatic functions. The variations in mRNA levels were assessed by means of the differential display technique [14], which allows the simultaneous evaluation of the iron-induced up- or down-regulation of a number of genes.

2. Materials and methods

2.1. Cell cultures

HepG2 cells were grown as previously described [15] and incubated with ferric ammonium citrate (FAC, 225 μg/ml, or 40 μg Fe/ml, 716 μM) for 3–7 days ± α-tocopherol (vitamin E, 100 μM), or treated with 100 μM H_2O_2 or 15 μM 4-hydroxy-2,3-nonenal (4-HNE) for 7 or 24 h. Cell viability was determined by means of trypan blue exclusion and LDH release.

2.2. Iron deposition staining

HepG2 cells were rinsed four times with phosphate-buffered saline and then stained according to Scheuer et al. [16].

2.3. Lipid peroxidation assay

Lipid peroxidation products were measured using the Bioxytech LPO-586 kit (Oxis International, Portland, OR, USA) [17] on 200 μl of cell supernatant to which butylated hydroxytoluene was added to a final concentration of 5 mM.

2.4. Total and poly(A)$^+$ RNA isolation

Total RNA was extracted from the control and FAC-stimulated HepG2 cells using the Trizol reagent (Life Technologies, Milan, Italy). Poly(A)$^+$ RNA was isolated using oligo $(dT)_{25}$ magnetic beads (Dynabeads oligo $(dT)_{25}$, Dynal, Oslo, Norway) according to the manufacturer's instructions.

2.5. cDNA synthesis and differential display

Total RNA was treated with 1 U RNase-free DNase/μg RNA (Life Technologies, Milan, Italy) and then retrotranscribed. Differential display PCR reactions were performed with a combination of 10 arbitrary and nine anchored oligo (dT) primers from the Delta RNA fingerprinting kit (Clontech, Palo Alto, CA, USA). The PCR reaction products were separated on 5% denaturing polyacrylamide sequencing gels, transferred onto 3MM paper, dried under vacuum and exposed for 18 h. The products were considered to be differentially expressed

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Abbreviations: IRE, iron regulatory element; 4-HNE, 4-hydroxy-2,3-nonenal; Apo B100, apolipoprotein B100 mRNA; FAC, ferric ammonium citrate; TfR, transferrin receptor

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only if the same variations were observed in reactions performed on two different cDNAs.

2.6. Cloning and selection of cDNA fragments

Differentially expressed bands were cut from the dried gels, eluted by boiling, re-amplified and subcloned into the P-MOS vector (Amersham, Milan, Italy). Twenty independent clones were randomly chosen for each fragment; the differential clones were selected using a modification of the reverse Northern technique [18] and then sequenced using the Amplicycle sequencing kit (Perkin Elmer, Monza, Italy). The gene database searches were made at the National Center for Biotechnology Information using the BLAST network service.

2.7. Northern blot analysis

Northern blots with total (40 μg) or poly(A)$^+$ (3 μg) RNA were performed as previously described [15]. The probes derived from differential display, human TfR [19], transferrin [20], H ferritin [21], human albumin, β-actin and ubiquitin cDNAs (ATCC, MD, USA) were labeled with [^{32}P]dCTP (800 mCi/mmol, Amersham, Milan, Italy) by random priming (Megaprime kit, Amersham, Milan, Italy). Autoradiography was quantified by computer-assisted densitometry.

2.8. Protein extraction and Western blot analysis

The proteins were extracted using the Trizol reagent (Life Technologies, Milan, Italy) and quantified by the Bradford method [22]. The proteins (10 μg/lane) were separated on a 12% SDS–PAGE gel under reducing conditions [23], and electroblotted onto ECL membranes (Amersham, Milan, Italy). Anti-human aldose reductase (gift of Dr. Peter Kador), anti-β-actin (Sigma, Milan, Italy) and anti-human apolipoprotein B100 (Apo B100) (Boehringer, Milan, Italy) were used at a 1:1000 dilution, and the horseradish peroxidase-labeled anti-rabbit IgG, anti-mouse IgG (Amersham, Milan, Italy) and anti-goat IgG (Sigma, Milan, Italy) at a dilution of 1:5000. The proteins detected using the specific antibodies were visualized by chemoluminescence (ECL, Amersham, Milan, Italy), and the bands quantified by computer-assisted densitometry.

2.9. Data analysis

Data comparison was performed using the Student–Newman–Keuls test.

3. Results

Seven days FAC treatment at a final Fe concentration of 40 μg/ml (712 μM) induced iron overload as assessed by Pearls' staining (50% positive cells) without affecting cell viability (>90%). Moreover, there was a more than 90% reduction in the level of TfR mRNA after 1 week of iron overloading, an effect that was already present after 3 days of treatment (Fig. 1, lanes Fe+/Vit. E−). Iron supplementation did not have a toxic effect on the HepG2 cells, since there was no significant variation in albumin mRNA levels (Fig. 1), being, at 7 days, 92 ± 3.2% and 89.5 ± 4.5% for Fe+/Vit. E− and Fe+/Vit. E+, respectively. Moreover, no significant decrease

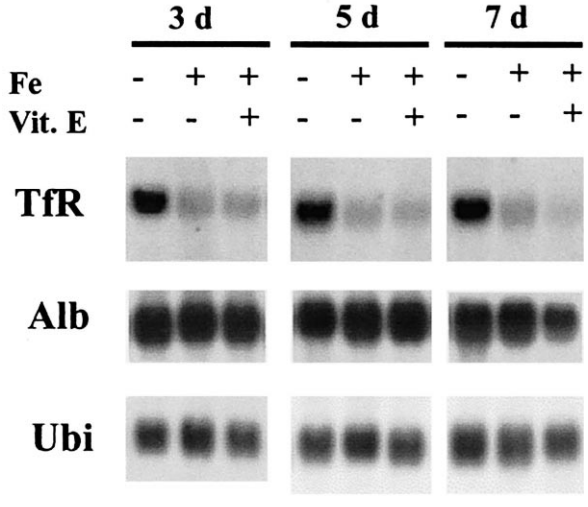

Fig. 1. TfR and albumin (Alb) expression in response to iron treatment. Total RNA isolated from HepG2 cells left untreated (Fe−/Vit. E−), or cultured in the presence of iron (Fe+/Vit. E−) or iron and vitamin E (Fe+/Vit. E+) for 3, 5 and 7 days (d) was hybridized with TfR, albumin and ubiquitin (Ubi) cDNA. Autoradiographies are representative of at least three independent experiments.

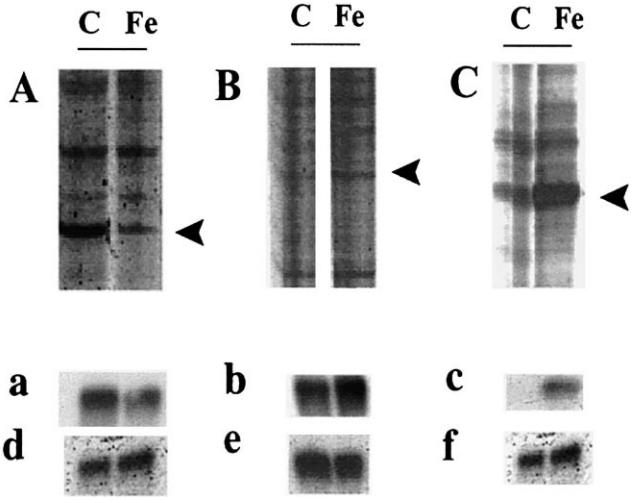

Fig. 2. Differential display and Northern blot analysis of differentially expressed clones. Autoradiography of polyacrylamide denaturing gels with differential display PCR products obtained from control (C) or iron-overloaded cell cDNA (Fe) (A, B and C). The arrows indicate the differential bands which corresponded to Apo B100 (A), semaphorin cd100 (B) and aldose reductase (C). Northern blot hybridization on poly(A)$^+$ RNA with Apo B100 (a), semaphorin cd100 (b), aldose reductase (c) and ubiquitin cDNAs (d–f).

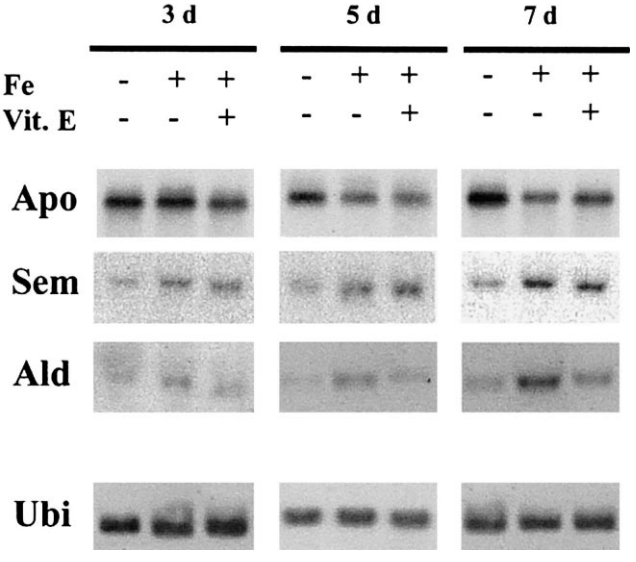

Fig. 3. Northern blot analysis of Apo B100, semaphorin cd100 and aldose reductase expression after iron treatment ± vitamin E. Toal RNA isolated from HepG2 cells left untreated (Fe−/Vit. E−), or cultured in the presence of iron (Fe+/Vit. E−) or iron and vitamin E (Fe+/vit. E+) for 3, 5 and 7 days (d) was hybridized with Apo B100 (Apo), semaphorin cd100 (Sem), aldose reductase (Ald) and ubiquitin (Ubi) cDNAs. Autoradiographies are representative of at least three independent experiments.

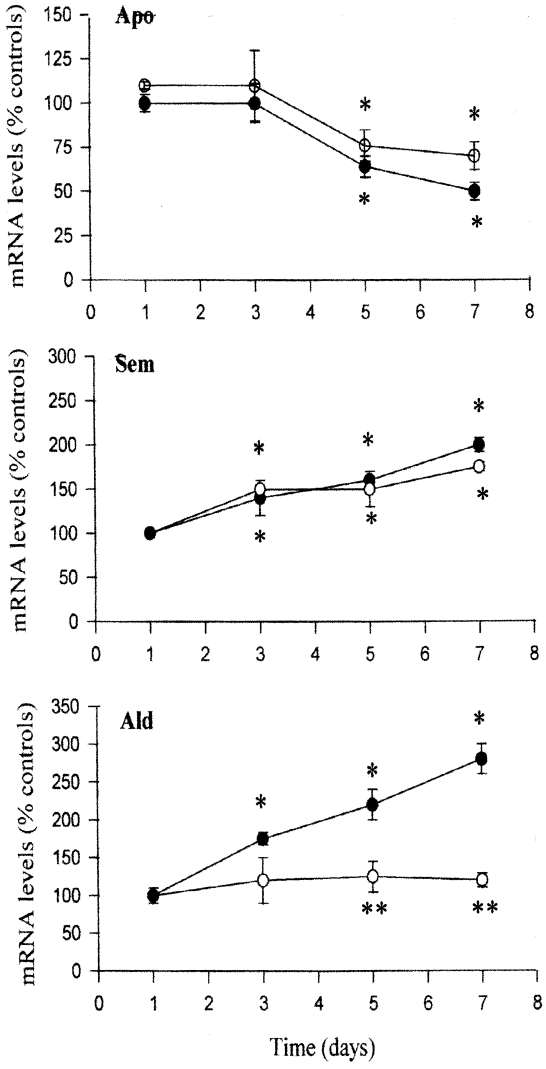

Fig. 4. Quantitative analysis of Apo B100, semaphorin cd100 and aldose reductase mRNA levels after iron treatment ± vitamin E. HepG2 cells were left untreated (Fe−/Vit. E−), or cultured in the presence of iron (Fe+/Vit. E−) (●) or iron plus vitamin E (Fe+/Vit. E+) (○) for 1, 3, 5 and 7 days. The mRNA levels of Apo B100 (Apo), semaphorin cd100 (Sem) and aldose reductase (Ald) were quantified by densitometry and normalized using ubiquitin and β-actin hybridizations. The mRNA levels from untreated cells were arbitrarily set at 100%. The results are the mean values ± S.E.M. of at least three independent experiments. *$P < 0.05$ vs. controls, **$P < 0.05$ vs. iron overload.

was observed in the mRNA levels of transferrin and H ferritin (data not shown).

The RNAs from control and iron-loaded HepG2 cells were analyzed using the differential display technique and, after reverse Northern screening, 16 bands were identified as being differentially represented: four of these bands were more abundant in the controls and the remaining 12 in the iron-overloaded samples. Of the final 16 independent clones, two corresponded to different regions of Apo B100 cDNA (from 5614 to 6202 bp and from 9885 to 10030 bp), two to semaphorin cd100 cDNA (from 741 to 1033 bp and from 3480 to 3886 bp), and one to the 3′ end region of the aldose reductase transcript (from 1179 to 1335 bp). The other 11 clones showed homology with expressed sequence tags, and are currently under investigation.

The differential display reactions identifying the three known genes are shown in Fig. 2. Northern blot hybridization on poly(A)$^+$ RNA confirmed the differential representation of all three mRNAs (Fig. 2a–c), with an increase in semaphorin cd100 and aldose reductase mRNAs and a decrease in the Apo B100 transcript.

The effects of iron overloading (40 μg/ml of Fe) at different times (3, 5 and 7 days) on Apo B100, semaphorin cd100 and aldose reductase mRNAs, as well as those of the concomitant administration of vitamin E, are shown in Fig. 3. The intensity of the hybridized bands was normalized for differences in loading and the effect of the iron overload is graphically represented in Fig. 4. An initial increase in aldose reductase and semaphorin cd100 mRNAs was evident after 3 days of iron treatment; the effect on Apo B100 appeared after 5 days. However, the effect of iron overload on gene expression was more pronounced after 7 days of treatment (Fig. 3, 7d, lanes Fe+/Vit. E−, and Fig. 4). Iron treatment reduced the level of Apo B100 mRNA by approximately 50%, whereas the levels of semaphorin cd100 and aldose reductase mRNAs respectively doubled and tripled ($P < 0.05$ vs. controls). Parallel treatment with ammonium citrate alone was not able to induce changes in the mRNA levels of any of the genes analyzed (data not shown).

After 7 days of iron treatment, lipid peroxidation product levels of 9.8 ± 1.1 μM were observed, but none was detectable in the controls or the supernatants of the cells treated with iron and vitamin E, thus demonstrating both the presence of iron-induced lipid peroxidation and the efficacy of the antioxidant treatment. Antioxidant had no effect on albumin mRNA or on the iron-induced reduction in TfR mRNA (Fig. 1, lanes Fe+/Vit. E+), whereas it almost completely prevented the induction of aldose reductase mRNA at 3, 5 and 7 days (Fig. 3, lanes Fe+/Vit. E+ and Fig. 4), without any significant effect on the iron-induced modification in Apo B100 and semaphorin cd100 mRNA levels ($P > 0.05$ vs. iron treatment alone).

The changes in aldose reductase and Apo B100 protein levels paralleled the variations in mRNA at 3, 5 and 7 days. Semaphorin cd100 protein level was not assessed due to the unavailability of specific antibodies. Vitamin E treatment had

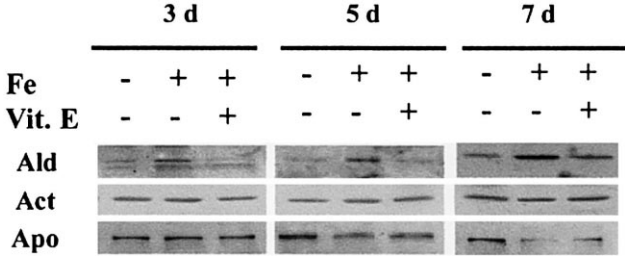

Fig. 5. Western blot analysis of aldose reductase (Ald) and Apo B100 (Apo) expression after iron treatment ± vitamin E. The HepG2 cells were left untreated (Fe−/Vit. E−), or cultured in the presence of iron (Fe+/Vit. E−) or iron plus vitamin E (Fe+/Vit. E+) for 1, 3, 5 and 7 days (d). Proteins from the cell extracts (for aldose reductase and β-actin analysis) or culture media (for Apo B100) were separated on SDS–PAGE, electroblotted on a membrane and probed with specific antibodies. Results are representative of three independent experiments.

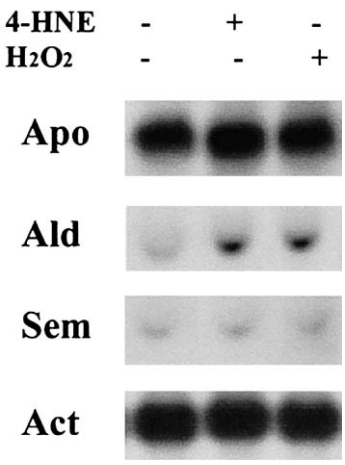

Fig. 6. Northern blot analysis of Apo B100, semaphorin cd100 and aldose reductase expression after treatment with 4-HNE or H_2O_2. Total RNA extracted from HepG2 cells cultured in control media (4-HNE−/H_2O_2−) or in the presence of 4-HNE (15 μM, 4-HNE+/H_2O_2−) or H_2O_2 (100 μM, 4-HNE−/H_2O_2+) for 24 h was hybridized with the Apo B100 (Apo), semaphorin cd100 (Sem), aldose reductase (Ald) and β-actin (Act) cDNAs. Autoradiographies are representative of at least three independent experiments.

no significant effect on Apo B100 protein levels, but prevented aldose reductase induction (Fig. 5, lanes Fe+/Vit. E+).

In order to assess the role of oxidative stress and lipid peroxidation products in the observed variations in gene expression, HepG2 cells were stimulated for 7 and 24 h with hydrogen peroxide and 4-HNE. No changes in Apo B100 or semaphorin cd100 mRNA were observed, whereas treatment with 4-HNE or H_2O_2 for 24 h (but not for 7 h) induced aldose reductase mRNA (Fig. 6).

4. Discussion

Iron can control the expression of the genes directly involved in its metabolism, such as TfR and ferritin [3]; this regulation takes place at the post-transcriptional level and, in the case of TfR, involves mRNA degradation.

However, the use of differential display allowed us to identify the effects of iron overload on the expression of genes other than those directly involved in its metabolism. Treatment with FAC induced a large increase in intracellular iron, and a significant reduction in TfR mRNA, without impairing cell viability or hepatic synthesis. The lack of induction of ferritin mRNA confirms also what was previously observed by Hubart et al. [4], thus supporting the presence of a post-transcriptional regulation of ferritin synthesis in these cells.

The differential display analysis identified the modulation of several transcripts, including Apo B100, semaphorin cd100 and aldose reductase.

Apo B100 is the major protein component of VLDL and LDL, and is essential for maintaining normal lipid metabolism [24]. In our experimental system, Apo B100 mRNA decreased by 50% after 1 week of iron treatment, and parallel changes were observed at the protein level. These data support the presence of an interaction between iron and lipid metabolism, as has also recently been reported by Brunet et al. [25], by using an in vivo model.

It is known that iron overload can induce free radical formation and lipid peroxidation in the liver [26], and in agreement to what was previously reported [27], we found that peroxidation products reached levels of 10 μM in the presence of iron, whereas vitamin E treatment inhibited lipid damage. The addition of the antioxidant had only a slight effect on the iron-induced reduction in Apo B100 mRNA and protein. Moreover, the reduction in Apo B100 mRNA was not due to a generalized toxic effect, as previously observed in iron-overloaded rats where hepatic functions were not altered [28].

Conversely, iron loading led to an increase in the mRNA levels of semaphorin cd100 and aldose reductase genes. Semaphorin cd100 is a recently identified gene whose expression has previously been described in other tissues, but not in the liver [29]; we found a very low but detectable level of mRNA in untreated HepG2 cells, which was increased by iron stimulation. As in the case of Apo B100, this effect was not altered by the addition of vitamin E, thus suggesting that free radicals and lipid peroxidation are not directly involved in the modulation. Semaphorin cd100 expression promotes B-cell survival [29], but its precise function still remains to be defined in other tissues. It is interesting to note that overexpression of semaphorin E, another protein belonging to the same family, could be related to cell defence mechanisms [30,31].

Iron overloading tripled the mRNA and protein levels of aldose reductase, an NADPH-dependent aldo-keto reductase that converts glucose to sorbitol in the polyol pathway, but can also reduce 4-HNE, one of the major products of lipid peroxidation [32]. It has been shown that 4-HNE or H_2O_2 treatment of smooth muscle vascular cells can lead to a significant increase in aldose reductase mRNA [33,34]. These findings suggest that aldose reductase may act as a detoxifying enzyme. In this regard, the results of the present study indicate that aldose reductase expression may be a response to the lipid peroxidation induced by iron overload in HepG2 cells. This is supported by the observed ability of vitamin E to prevent, and 4-HNE to induce, the increase in aldose reductase mRNA.

Conversely, a mechanism independent from the generation of free radical formation and lipid peroxidation can be hypothesized for either Apo B100 or semaphorin cd100.

In conclusion, there are a number of pathways by means of which iron overload can induce variations in the mRNA levels of genes other than those directly involved in its metabolism. Further studies are necessary to assess whether iron acts on transcription rates (by activating NF-kB or other factors) or mRNA stability.

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