Oxidative stress and pro-apoptotic conditions in a rodent model of Wilson’s disease

Alberta Samuele, Anna Mangiagalli, Marie-Thérèse Armentero, Roberto Fancellu, Eleonora Bazzini, Mariapia Vairetti, Andrea Ferrigno, Giuseppe Nappi, Fabio Blandini

Abstract

Wilson’s disease (WD) is an inherited disorder, characterized by selective copper deposition in liver and brain, chronic hepatitis and extra-pyramidal signs. In this study, we investigated changes of biochemical markers of oxidative stress and apoptosis in liver, striatum and cerebral cortex homogenates from Long–Evans Cinnamon (LEC) rats, a mutant strain isolated from Long Evans (LE) rats, in whom spontaneous hepatitis develops shortly after birth. LEC and control (LE) rats at 11 and 14 weeks of age were used. We determined tissue levels of glutathione (GSH/GSSG ratio), lipid peroxides, protein-thiols (P-SH), nitric oxide metabolites, activities of caspase-3 and total superoxide-dismutase (SOD), striatal levels of monoamines and serum levels of hepatic amino-transferases. We observed a decrease of protein-thiols, GSH/GSSG ratio and nitrogen species associated to increased lipid peroxidation in the liver and striatum – but not in the cerebral cortex – of LEC rats, accompanied by dramatic increase in serum amino-transferases and decrease of striatal catecholamines. Conversely, SOD and caspase-3 activity increased consistently only in the cortex of LEC rats. Hence, we assume that enhanced oxidative stress may play a central role in the cell degeneration in WD, at the main sites of copper deposition, with discrete pro-apoptotic conditions developing in distal areas.

Keywords: Apoptosis; Copper-toxicosis; Long Evans Cinnamon; Peroxidation; Cell degeneration

1. Introduction

Wilson’s disease (WD) is a recessive autosomal inherited disorder of copper transport and metabolism, with an estimated prevalence of approximately 1 in 50,000 live births. The disease, also termed hepatolenticular degeneration, is characterized by deficient ceruloplasmin biosynthesis and marked impairment in biliary copper excretion, resulting in severe tissue accumulation of the metal, selectively in the liver and central nervous system (predominantly in basal ganglia, subthalamic nuclei, gray and white matter) [1,2]. Clinical phenotype includes recurrent or fulminant hepatitis, leading to chronic cirrhosis or hepatocarcinoma, paralleled by a variety of neurological signs, usually latent before the third decade of life, such as depression, schizophrenia-like disorders, dystonia, pseudosclerosis, akinetic rigid syndrome and Parkinsonian symptoms [3]. The gene (ATP7B) defective in WD has been recently identified on chromosome 13, and found to encode an intracellular trans-membrane copper transporter, belonging to the large family of cation transporting P-type ATPases, mainly expressed in hepatocytes and neurons.

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[4]. On this regard, it has been recently reported that the expression of transcript and protein in the liver of transgenic rats results in restoration of hepatic dysfunctions and biliary copper excretion [5]. Furthermore, recent molecular genetic analysis of patients affected by aceruloplasminemia, characterized by low serum ceruloplasmin levels and basal ganglia symptoms, revealed the presence of mutations in the ceruloplasmin gene and suggested interactions between clinical phenotypes and metal metabolism disorders [6].

Copper is an essential trace element, required for the proper function of important enzymes, such as cytochrome c oxidase, which catalyzes the reduction of oxygen to water in the mitochondrial respiratory chain, Cu/Zn superoxide-dismutase (Cu/Zn SOD) – one of the main scavengers of reactive oxygen species (ROS) – or dopamine-β-hydroxylase, a crucial enzyme in the catecholamine biosynthetic pathway. Furthermore, copper-binding proteins play a key role in the establishment and maintenance of metal-ion homeostasis [7]: excess copper seems to compete with zinc absorption, which is essential for the antioxidant activity of Cu/Zn SOD. Moreover, there is increasing evidence that ceruloplasmin deficiency and subsequent reduction of its ferroxidase activity may result in iron transport impairment and accumulation of this metal in various organs [8]. This phenomenon may, in turn, affect the activity of enzymes such as monoamine oxidase (MAO) or catecholamine-O-methyltransferase (COMT), which metabolize monoaminergic neurotransmitters [3]. Hence, in inherited disorders of copper metabolism, such as WD or Menkes’ disease, metal-ion homeostasis appear severely dysregulated, thus accounting for most of the neurological symptoms documented [9,10].

Aberrations in copper dynamics may create favorable conditions for superoxide-yielding redox cycling, and, therefore, oxidative damage to susceptible regions, such as liver or brain, where easily oxidizable substrates abound (e.g., membrane polyunsaturated lipids), and redox-based neurochemical reactions predominantly occur, leading to increased ROS production [11–13].

Analogously to WD, genetically determined tissue deposition of copper is observed in Long–Evans Cinnamon (LEC) rat, an inbred mutant strain isolated from Long–Evans (LE) rat, designated from its peculiar cinnamon-like coat color, in which spontaneous hepatitis shortly develops after birth. Genetic analysis recently showed that Atp7b, the rat orthologue of ATP7B, has a mutation in the LEC rat, demonstrating that these rats are a valid model of WD [14,15]. Indeed, in LEC rats, the human disease is mimicked in terms of copper accumulation in the liver and impaired hepatic excretion of copper into blood and bile [16]. Affected rats manifest, thus, jaundice, elevated serum levels (up to 500 IU/L) of alanine and aspartate aminotransferases (ALT, AST). In the brain, inflammatory tissue infiltrates, associated with increased copper and iron concentrations, and histological changes in monoaminergic neurons have been documented, as well as severe suppression of feeding regulation center in the hypothalamus [17–20]. Approximately 40% of these animals succumb of fulminant hepatitis within 1 week of the appearance of jaundice [21,22].

Oxidative stress and apoptosis may play a crucial role in inducing liver and brain damage in WD; recent investigations revealed considerable increases in DNA single strand breaks and 8-hydroxy-deoxy-guanosine levels – two indices of DNA oxidative damage – in brain, liver and kidney of affected animals [11–13,23,24]. In addition, other authors suggested that lipid peroxidation might be involved in Cu-mediated toxicity in LEC liver [11,12].

The aim of our study was, therefore, to test the role of oxidative stress and pro-apoptotic conditions in WD-related liver and brain damage, using the LEC model. For this purpose, we evaluated NO end-product levels, lipid peroxidation and caspase-3 activity, as well as SOD activity, GSH/GSSG ratio and protein-thiols (P-SH) content in liver and selected brain areas (striatum and cerebral cortex). Finally, hepatic function and monoaminergic neurotransmission were evaluated by determination of serum aminotransferase levels and striatal catecholamine content, respectively.

2. Materials and methods

Two groups of male LEC and LE rats (Charles River, Calco, LC, Italy) were examined in this study: seven at 11 weeks of age and twelve at 14 weeks. Animals were housed two per cage, maintained in air-conditioned environment on a 12-h light/dark schedule at 20–22 °C, and had free access to food and water. During the housing period, the health of animals was regularly monitored, and all procedures were conducted in accordance with approved animal care guidelines of European Convention for Animal Care and Use of Laboratory Animals (86/609/ EEC).

Rats were anesthetized by exposure to carbon-dioxide and sacrificed by decapitation, then truncal blood was immediately collected to determine the serum levels of ALT and AST, using an automated analyzer (COBAS Integra, Roche Diagnostics GmbH, Mannheim, Germany), in accordance with the International Federation of Clinical Chemistry (IFCC) method.

Liver, striatum and cerebral cortex were quickly removed, frozen and stored at −80 °C. The day of the assay, tissue samples were homogenized by sonication (VibraCell, Sonics and Materials, Inc., Danbury, CT, USA) in phosphate-buffered saline (PBS) pH 7.4 (Sigma-Aldrich, St. Louis, MO; USA), then centrifuged (20,000 × g, at 4 °C). Supernatants were used to quantify nitrite and nitrate concentration, GSH and GSSG levels, lipid-peroxides and protein-thiols (P-SH) content. Enzymatic activities of caspase-3 and total superoxide-dismutase (SOD) were also
Age-related changes in the activities of superoxide-dismutase (SOD) and Caspase-3, and in the concentration of nitrite/nitrate in the liver, striatum and cerebral cortex of LE and LEC rats (mean ± S.D.)

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>SOD U/mg prot</th>
<th>Caspase-3 pmol AMC/mg prot</th>
<th>Nitrite/Nitrate pmol/mg prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.97 ± 0.18</td>
<td>0.68 ± 0.46</td>
<td>85.22 ± 39.36</td>
</tr>
<tr>
<td>14</td>
<td>0.57 ± 0.18</td>
<td>0.93 ± 0.50</td>
<td>126.60 ± 54.32</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2.61 ± 1.02</td>
<td>1.21 ± 0.31</td>
<td>375.17 ± 62.22</td>
</tr>
<tr>
<td>14</td>
<td>1.25 ± 0.24</td>
<td>1.29 ± 0.50</td>
<td>250.56 ± 47.32</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.62 ± 0.12</td>
<td>0.87 ± 0.20</td>
<td>92.42 ± 31.72</td>
</tr>
<tr>
<td>14</td>
<td>0.57 ± 0.16</td>
<td>0.94 ± 0.24</td>
<td>154.22 ± 34.53</td>
</tr>
</tbody>
</table>

a P < 0.005.
b P < 0.05 vs. LE rats.
c P < 0.01.

3. Results

3.1. Liver

As shown in Table 1, a significant increase in hepatic SOD activity was observed in LEC rats at the 14th week, after a slight initial decline. Conversely, a marked reduction of NO metabolites emerged in all affected animals, while no significant changes were shown for caspase-3 activity. Table 2 indicates that both P-SH content and GSH/GSSG ratio remained significantly lower in the liver of LEC rats throughout the experiment, while hepatic TBARS levels were markedly increased in mutants, compared to controls, at the 14th week. Serum levels of AST and ALT were massively increased in LEC compared with LE rats, already at the 11th week, showing further dramatic elevation in the 14-week-old animals (Table 3).

3.2. Striatum

Significant reductions in SOD and caspase-3 activities, and in nitrate/nitrite levels were found in the striatum of mutants compared to controls, but only at the first time point (Table 1). Similarly, both DA and NE striatal levels were lower in mutants than in healthy animals at the 11th week, while this difference tended to disappear at the 14th week (Table 4). As shown in Table 2, striatal TBARS content was significantly higher in LEC than in LE rats in the second phase of the study; this was associated with a slight, but significant decrease in both P-SH levels and GSH/GSSG ratio.

3.3. Cerebral cortex

As shown in the Table 1, SOD activity in the cortex of mutant animals was elevated at each time point compared with the normal controls; caspase-3 activity and nitrite/nitrate levels were higher in LEC than LE rats, although differences between the two groups were significant only at the 14th week. Conversely, no relevant differences between LE and LEC animals were detected concerning...
cortical P-SH, GSH/GSSG, and TBARS levels, although their contents remained moderately lower in mutants (Table 2).

4. Discussion

Although the molecular processes underlying copper-induced cytotoxicity remain rather enigmatic, it has been postulated that enhanced oxidative stress, possibly activating the apoptosis cascade, may play a central role in a disorder strictly related to copper accumulation, such as WD [13]. The present study was therefore undertaken to elucidate the role of these processes in the pathogenetic mechanisms underlying WD, using a rodent model.

The hepatic damage, a typical feature of LEC rats, was reflected by massive increases in the serum levels of AST and ALT, which paralleled the progression of the degenerative processes. In these animals, we observed a consistent decrease in the levels of endogenous antioxidant agents, GSH/GSSG ratio and P-SH amount, in particular in the striatum and liver, which were associated with an increased rate of membrane lipid peroxidation, as expressed by the enhanced TBARS production. Less consistent were the changes in terms of SOD activity, which was increased in the cortex of LEC animals, at both 11- and 14-week-old LEC rats. The striatal involvement was mirrored, at least in younger animals, by the decrease in DA and NE levels observed in the LEC group. Curiously, the differences between the groups disappeared at the 14th week, due to a decrease of DA and NE levels in the striata of LE animals. The phenomenon (which tended to involve also other variables of the study) was clearly age-related and may, indeed, be linked to the sexual maturation process taking place in rats of this age. In particular, it has been shown that the transition from a developmental to a post-developmental stage of life is accompanied by consistent changes in the central monoaminergic tone, represented — for example — by a progressive decrease in the release of DA and NE from selected brain areas, including striatum and locus coeruleus, which begins at the 4th–5th week of age and tend to stabilize after the 16th week [35,36].

Hydroxyl radicals are generated in liver during the cyclic regeneration of GSH from GSSG, a process catalyzed by free circulating copper [12]. Elevated copper levels cause relevant changes in the intracellular structure of hepatocytes, particularly at the level of mitochondria, the main cellular source of ROS [11]. Excessive intra-mitochondrial copper import may therefore disrupt normal oxidative balance and elicit pro-apoptotic conditions. On this regard, our data are not conclusive, in fact, LEC animals showed increased activity of apoptotic effector caspase-3, only in the cerebral cortex, which was virtually unaffected by pro-oxidant changes. On the other hand, it may be hypothesized that, since the cerebral cortex resides rather distally from the regions characterized by preferential copper deposition, subtle pro-apoptotic conditions may develop at this level, as opposed to the necrotic processes occurring in the liver and striatum.

Concerning the nitrite/nitrate results, it has to be remarked that NO acts as a neuromodulator in the central nervous system, being implicated in development and...
several neuronal mechanisms of synaptic transmission and plasticity, underlying learning and memory, as well as in the maintenance of basal vascular tone and regulation of cerebral microcirculation. NO also acts as a free radical, capable of binding to O$_2$ and yielding peroxy-nitrate anion, which is a powerful nitrating agent able to oxidize and modify proteins, lipids and DNA, as well as to deplete antioxidant defenses (nitrosative stress) [24]. In addition, NO-synthase activation seems to be highly influenced by circulating redox-active metals, like copper. However, depending upon cellular environments, NO has been described to exert either neuroprotective or neuro-destructive effects. Our data showed that LEC liver and striatum underwent a strong depletion of NO metabolites, compared to those of LE animals, which may be related to the fact that both copper and iron, due to their versatile coordination chemistry and reactivity, participate in a variety of electron-transfer reactions employing NO, and may readily bind to nitric compounds, presumably improving a sort of denitrification process. This term generally refers to an anaerobic four consecutive reduction steps process, typical of microbial species, which involves an anion, which is a powerful nitrating agent able to oxidize and modify proteins, lipids and DNA, as well as to deplete antioxidant defenses (nitrosative stress) [24].

In conclusion, our data would confirm the hypothesis that disturbance in cupro-enzyme regulation may enhance oxidative stress and pro-apoptotic conditions in hepatocytes and neurons, presumably interfering with anti-oxidant scavenger systems, NO metabolism and monoaminergic neurotransmission. Additional morphological, molecular and immunohistochemical explorations would have a relevant impact on understanding neuron apoptosis accompanying WD pathogenic mechanisms and may accelerate development and testing of new therapeutic approaches [41,42].

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References


