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# ALDEHYDE-PROTEIN ADDUCTS IN THE LIVER AS A RESULT OF ETHANOL-INDUCED OXIDATIVE STRESS

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## 1. ABSTRACT

A number of systems that generate oxygen free radicals and reactive aldehydic species are activated by excessive ethanol consumption. Recent studies from human alcoholics and from experimental animals have indicated that acetaldehyde and aldehydic products of lipid peroxidation, which are generated in such processes, can bind to proteins forming stable adducts. Adduct formation may lead to several adverse consequences, such as interference with protein function, stimulation of fibrogenesis, and induction of immune responses. The presence of protein adducts in the centrilobular region of the liver in alcohol abusers with an early phase of histological liver damage indicates that adduct formation is one of the key events in the pathogenesis of alcoholic liver disease. Dietary supplementation with fat and/or iron strikingly increases the amount of aldehyde-derived epitopes in the liver together with promotion of fibrogenesis.

## 2. INTRODUCTION

Evidence continues to grow indicating that reactive aldehydic products resulting from ethanol metabolism and ethanol-induced oxidative stress play a pivotal role in the pathogenesis of alcoholic liver injury (1-6). Reactive aldehydes and hydroxyl radicals, which may be generated during periods of heavy ethanol intake, are known for their ability to attack amino acid residues of proteins thereby forming both stable and unstable adducts with proteins and cellular constituents (1, 7-14). As a consequence, cellular functions may become disturbed together with damage to proteins, nucleic acids and lipids (9, 15-17). Several different types of adducts have been recently identified. The purpose of the present communication is to address the question what are the real adducts formed *in vivo* based on recent experiments carried out both in alcoholic patients and in experimental animals. The relationship between adduct formation and alcohol toxicity is also discussed.

## 3. FORMATION OF PROTEIN-ALDEHYDE ADDUCTS

### 3.1. Reactive compounds generated during ethanol metabolism

Adducts of proteins with *acetaldehyde*, the first metabolite of ethanol, have been described in a number of studies (table 1). Acetaldehyde forms adducts primarily via binding to reactive lysine residues of preferred target proteins (1,6,7,10,18). While the data on the reactivity of acetaldehyde at physiologically relevant concentrations have remained controversial, it appears that among acetaldehyde-exposed proteins those with abundant amounts of reactive lysine residues are readily modified even at low concentrations of acetaldehyde under appropriate reducing conditions (19,20). On the other hand, even in the absence of reducing agents stable cyclic imidazolidinone structures are generated as a result of a reaction between acetaldehyde and the free alpha-amino group of the aminoterminal valine of hemoglobin (21,22).

Aldehydic products of lipid peroxidation, such as *malondialdehyde (MDA)* and *4-hydroxynonenal (HNE)*, also form Schiff's base adducts with proteins (table 1). MDA is a highly reactive dialdehyde originating from nonenzymatic lipid peroxidation of a variety of unsaturated fatty acids, from lipid peroxidation that occurs during phagocytosis by monocytes and from arachidonic acid catabolism in thrombocytes (23,24). The free radical-

**Table 1.** Reactive compounds generating protein adducts in alcohol abusers.

| Compound | Abbreviation | Reference |
|---|---|---|
| Acetaldehyde | AA | Nomura and Lieber 1981,[78] Medina *et al*. 1985,[79] Israel *et al*. 1986,[10] Behrens *et al*. 1988,[33] Niemelä *et al*. 1991,[65] 1994,[28] 1995,[76] Lin *et al*. 1988,[11] Halsted *et al*. 1993,[67] Holstege *et al*. 1994,[66] Yokoyama *et al*. 1995[61] |
| Malondialdehyde | MDA | French *et al*. 1993,[80] Niemelä *et al*. 1994,[28] 1995,[76] Tsukamoto *et al*. 1995[69] |
| 4-hydroxynonenal | HNE | Kamimura *et al*. 1992, [81] French *et al*. 1993, [80] Niemelä *et al*. 1995,[76] Tsukamoto *et al*. 1995[69] , Chen *et al*. 1998[82] Ohhira *et al*. 1998[83] |
| Malondialdehyde-acetaldehyde hybrid | MAA | Tuma *et al*. 1996[31] Xu *et al*. 1998[59] |
| Hydroxyethyl radical | HER | Moncada *et al*. 1994,[13] Clot *et al*. 1995,[32] 1996,[14] 1997 [84]Albano *et al*. 1996[85] |

**Table 2.** Preferred target proteins for adduct formation in alcoholics.

| Protein | Reference |
|---|---|
| Erythrocyte membrane proteins | Gaines *et al*. 1977[86] |
| Hemoglobin | Stevens *et al*. 1981,[7] San George and Hoberman 1986,[21] Niemelä and Israel 1992,[62] Peterson and Scott 1989[87] |
| Albumin | Donohue *et al*. 1983,[18] Israel *et al*. 1986[10] |
| Tubulin | McKinnon *et al*. 1987,[37] Jennett *et al*. 1989,[20] Smith *et al*. 1989,[38] Tuma *et al*. 1991[39] |
| Collagens | Jukkola and Niemelä 1989,[88] Behrens *et al*. 1989[89] |
| Lipoproteins | Haberland *et al*. 1988,[26] Steinberg *et al*. 1989,[27] Palinski *et al*. 1990,[23] Wehr *et al*. 1993,[58] Lin *et al*. 1995,[12] Paradis *et al*. 1996[90] |
| Ethanol-inducible cytochrome P450IIE1 | Behrens *et al*. 1988,[33] Clot *et al*. 1996[14] |
| Ketosteroid reductase (37 kD) | Lin *et al*. 1988,[11] Zhu *et al*. 1996[36] |

mediated oxidation of long-chain polyunsaturated fatty acids leads to the production of 4-hydroxynonenal, which can react with the sulfhydryl groups of proteins through a Michael addition type of mechanism (23-25). Oxidative modification of proteins with MDA and HNE have been demonstrated to occur *in vivo* on arterial vessel walls of atherosclerotic lesions (26,27). Similar epitopes have also been found from the liver specimens of patients with alcoholic liver disease (28) and from animals with experimental iron overload (29,30).

Tuma and coworkers have recently demonstrated the formation of *hybrid adducts with acetaldehyde and malondialdehyde,* designated as *MAA adducts*, in livers of ethanol fed rats (31). Such hybrid adducts may act in a synergistic manner and may also be involved in the mechanisms for stabilization of protein adducts *in vivo* (31). In addition, the appearance of *hydroxyethyl radicals*, a reactive species resulting from ethanol during its oxidation in the presence of iron, has also been described from liver microsomes of ethanol-fed animals (13,32).

### 3.2 Proteins involved in adduct formation

There seems to be several preferred target proteins for aldehyde attack *in vivo* (table 2). The continuously growing list of the primary targets include erythrocyte membrane proteins, hemoglobin, albumin, tubulin, lipoproteins, and collagens. Not surprisingly, adduct formation with acetaldehyde, hydroxyethyl radicals and ethanol-metabolizing cytochrome P450IIEI enzyme, seem to occur *in vivo* (14,33). Cytochrome enzymes are also known to be involved in the formation of the reactive compounds during ethanol metabolism (14,32-35). A 37 kD protein which has repeatedly been reported as a preferential binding site for acetaldehyde in Western blot experiments from liver fractions was recently identified as ketosteroid reductase, which catalyzes the reduction of key intermediates in bile acid biosynthesis (36).

## 4. CLINICAL SIGNIFICANCE OF PROTEIN MODIFICATIONS IN ALCOHOLICS

### 4.1. Functional consequenses

Formation of protein adducts with reactive aldehydic products has provided a basis for new hypotheses to explain the pathogenesis of ALD (figure 1), which have been previously reviewed in detail by Tuma and Sorrell (6,9). Covalent binding to proteins is known to interfere with protein function particularly when there is a lysine residue in a functionally critical location, such as in tubulin and in lysine-dependent enzymes (9,20,37-41). Altered microtubule function may subsequently lead to an impairment in protein secretion and plasma membrane

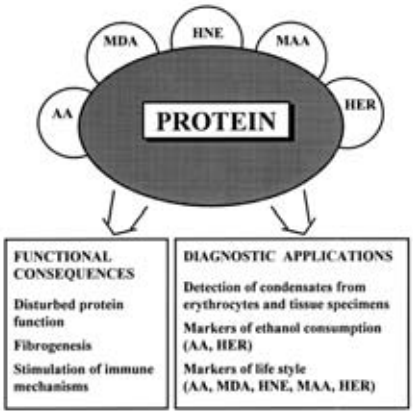


**Figure 1.** Schematic representation of various reactive components capable of attacking proteins to form adducts during ethanol metabolism. Protein binding with such compounds provides a basis to explain the pathogenesis of alcoholic liver disease and to diagnostic applications. See text for details. Abbreviations, AA, acetaldehyde, MDA, malondialdehyde, HNE, 4-hydroxynonenal, MAA, malondialdehyde-acetaldehyde hybrid adduct, HER, hydroxyethyl radical.assembly. Generation of reactive aldehydes may also contribute to ethanol-induced impairment in receptor-mediated endocytosis (42).

**4.2 Stimulation of fibrogenesis**

A number of cell culture studies have shown that aldehydic products derived from ethanol metabolism and lipid peroxidation can increase collagen mRNA levels and enhance the expression of connective tissue proteins (43-49). Acetaldehyde is able to increase the production of several extracellular matrix components (47,50). Reduction of adduct formation by scavengers of reducing equivalents has been shown to abolish such increases (44). Studies have further demonstrated that hepatic stellate cells (Ito cells) which are the primary source of extracellular matrix become readily activated under conditions involving enhanced oxidative stress and lipid peroxidation (49-52).

**4.3. Stimulation of immune reactions**

Aldehyde-protein adducts and hydroxyl radicals also stimulate immunological responses directed against the specific modifications of proteins (10,14,32,53-58). Studies have shown that chronic administration of ethanol to rats leads to the generation of circulating immunoglobulins with anti-acetaldehyde (10) adduct or anti-MAA-adduct (59) specificity. Similar immunoglobulins and autoantibodies recognizing cytochrome P450IIE1 hydroxyethyl radical adducts have also been found from the blood of human alcoholics (14,32). The highest titers of all such antibodies have been observed from patients with severe alcoholic liver disease (32,53-60). Characterization of the immunoglobulin isotypes involved in the above responses have revealed both IgA and IgG autoantibodies (32,56,60). In addition, immunization of experimental animals with aldehyde-protein conjugates has been shown to result in the production of antibodies recognizing protein-aldehyde condensates independently of the nature of the carrier protein (10,23). Such antibodies have considerable specificity towards aldehyde-lysine residues. Despite the small molecular weights of the aldehyde-amino acid condensates and the relatively small immunological differences between the various aldehyde-amino acid residues as haptens, antibodies with either anti-acetaldehyde or anti-malondialdehyde adduct specificity devoid of crossreactivity have been demonstrated in dot blot comparisons (28). It has further been shown that after immunization with acetaldehyde adducts, experimental liver disease can readily be induced by ethanol challenge to the immunized animals (61).

**4.4. Protein adducts as biological markers**

It is tempting to speculate that detection of ethanol-derived chemical condensation products could also serve as cumulative markers of ethanol intake (acetaldehyde adducts, hydroxyethyl radicals) or perhaps as markers of life style (epitopes induced by enhanced oxidant stress) (figure 1). In some studies, antibody-based detection of protein-acetaldehyde adducts has been used as an approach to study the existence of such condensates *in vivo*. However, despite the fact that increased concentrations of acetaldehyde adducts have been found to occur in the erythrocytes of alcohol consumers, lack of sensitivity of the adduct measurements from blood proteins has so far hampered their clinical use as alcohol markers (62-64).

Methods have also been developed for the detection of aldehyde-derived condensation products from tissue specimens (65). Immunohistochemical stainings for acetaldehyde-protein adducts from the liver of human alcohol abusers have revealed positive staining, which is restricted to the centrilobular region in the early phase of liver disease (65). Samples from heavy drinkers with no obvious clinical, biochemical or light microscopical signs of alcoholic liver disease were found to contain such epitopes primarily in the cytosol of hepatocytes. With progression of ALD the staining becomes more widespread. Studies by Holstege *et al* have further shown the occurrence of sinusoidal acetaldehyde adducts in alcoholic patients (66). Sinusoidal acetaldehyde adducts were also related to a poor prognosis of such patients (66). Liver specimens from human alcoholics have also been found to contain protein adducts with aldehydic products of lipid peroxidation (28). In comparisons relying on double immunofluorescence stainings, a significant colocalization between acetaldehyde and malondialdehyde adducts and histological tissue damage was observed (28). Moreover, a significant colocalization has been demonstrated between acetaldehyde adducts and the sites of collagen deposition in alcoholic liver disease (28,67).

## 5. PROTEIN ADDUCTS IN EXPERIMENTAL ANIMALS

**5.1. Rat model**

The perivenous staining pattern for acetaldehyde adducts in the liver after ethanol consumption has also been reproduced in experimental animals. Feeding of ethanol to Sprague-Dawley rats in a simultaneous pair feeding system (36% ethanol, 23% protein, 10% carbohydrate, and 32% fat) for four weeks resulted in small amounts of

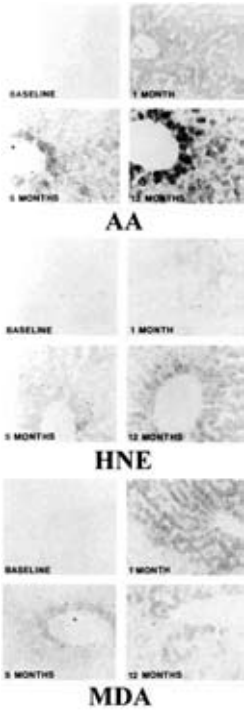


**Figure 2.** Sequential appearances of acetaldehyde (AA), malondialdehyde (MDA), and 4-hydroxynonenal (HNE) adducts in minipigs consuming ethanol. Biopsies were taken at 1,5, and 12 months after initiation of the ethanol diet. During follow-up abundant amounts of protein adducts were formed around the central vein coinciding with histopathological findings of steatonecrosis and focal inflammation and preceding fibrosis, which was evident at 12 months. See text for details. Immunoperoxidase staining, original magnification x 250. Reproduced with permission from Hepatology 22**,** 1200-1214 (76).

centrilobular and sinusoidal acetaldehyde adducts (28). Interestingly, acetaldehyde adducts were found to be most abundant in those animals, which also showed withdrawal symptoms during the course of the experiment indicating that individual high blood alcohol levels may be associated with increased amounts of adducts in tissues.

In recent experiments, where alcohol was administered to rats together with a high fat diet, distinct positive reactions for the different protein adducts were noted together with increased expression of cytochrome P450IIE1, although also with cytochrome 3A (68). Apparently, the high fat diet stimulates the formation of protein adducts with MDA and HNE. When ethanol-containing high-fat diet is further supplemented with iron a marked potentiation of adduct formation is seen together with strikingly elevated levels of serum liver-derived enzymes and progressive histopathology (69). The dietary iron supplementation to ethanol-diet in rats also results in the development of micronodular cirrhosis indicating that alcohol and iron together have a strong synergistic effect in producing liver pathology. Dietary supplementation with iron alone in amounts producing hemochromatosis in rats has also been found to result in the appearance of lipid-peroxidation derived aldehydes, although only in small amounts (29,30). Alcohol consumption may have an additive hepatotoxic effect also in human patients with iron burden (70-74).

### 5.2. Micropig model

A micropig model of alcohol-induced liver disease has recently been developed by Halsted and coworkers (67,75). Such micropigs consume ethanol voluntarily while demonstrating evidence of progressive hepatic injury, including steatonecrosis and fibrosis (67,76). The sequential appearances of acetaldehyde, MDA, and HNE adducts were examined from micropig liver biopsy specimens obtained at 1, at 5, and at 12 months after the initiation of the ethanol diet (figure 2). After 1 month on the ethanol-containing diet, AA and MDA adducts were observed in zone 3 hepatocytes colocalizing with each other and appearing together with increased serum concentrations of liver-derived enzymes (76). HNE adducts were usually less intense and more diffuse and were also seen in some biopsy specimens from control animals. The most intense reactions for each adduct were seen together with evidence of steatonecrosis and focal inflammation. In terminal biopsies at 12 months, perivenous fibrosis was present in most of the biopsy specimens, which had contained perivenous adducts of AA and MDA in the early phases of follow-up, suggesting that adduct formation precedes fibrogenesis in alcohol consumers. Recently, it was further shown that the formation of protein adducts is aggravated together with the induction of several cytochrome enzymes in castrated minipigs, suggesting that sex steroid hormones also play a role in the generation of liver toxicity through such mechanisms (77).

## 6. PERSPECTIVE

Recent work has rendered new insights on the origin and structure of protein adducts created as a result of excessive alcohol consumption. Analysis of studies indicates that several types of chemical condensation products with proteins are generated upon heavy alcohol intake. While the formation of such adducts seem to have an important pathogenic role in creating the adverse effects of ethanol in tissues, there may also be potential diagnostic applications for more specific detection of ethanol-induced diseases. However, the rate of formation and the relative importance of the various types of adducts needs to be addressed in future studies. This approach will eventually produce fruitful results in the next few years. It also remains to be established whether prevention of adduct formation could open new possibilities for therapeutic interventions in alcoholic patients.

## 7. ACKNOWLEDGMENTS

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