ARGinine CHALLENGE UNRAVELS PERSISTENT DISTURBANCES OF UREA CYCLE AND GLUCONEOGENESIS IN ABSTINENT ALCOHOLICS

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Abstract — Aims: Data on recovery from hormonal and metabolic sequelae of alcoholism in strictly controlled alcohol abstinence are mainly restricted to short-term abstinence. Our previous findings of persistently decreased plasma and urinary urea concentrations in long-term abstinent alcoholics prompted us to further elucidate this unexplained phenomenon. Methods: The response of circulating urea cycle metabolites and glucose-regulating hormones to an intravenous load (30 g) of arginine hydrochloride was investigated in abstinent male alcoholics (n = 14) after complete recovery of all routine liver parameters and compared with that in healthy male controls (n = 15). Results: The arginine challenge provoked (i) higher peak concentrations of arginine and increased arginine/ornithine and ornithine/citrulline ratios in the plasma of abstinent alcoholics; (ii) augmented plasma glutamine concentrations in alcoholics in the presence of comparable levels in both experimental groups of plasma glutamate, ammonia, and nitrate/nitrite; (iii) parallel increases in plasma urea concentrations over the respective baseline levels but distinctly higher urinary urea excretion in controls; (iv) a blunted blood glucose response to arginine in alcoholics together with a reduced insulin and glucagon surge; and (v) an elevated growth hormone peak as compared with controls. Conclusions: Application of an intravenous arginine challenge reveals profound and lasting metabolic and hormonal disturbances in abstinent alcoholics, affecting urea cycle and gluconeogenesis. The common denominator of many of these changes may be an acquired irreversible deficiency in cellular energy regulation.

INTRODUCTION

Alcohol dependence is an as yet incurable human brain disease with the molecular mechanisms of irreversibility still widely unknown. In fact, alcoholism causes a variety of profound and lasting hormonal and metabolic disturbances but data on their recovery in strictly controlled alcohol abstinence remain scarce. Careful follow-up of the long-term regeneration of peripherally accessible parameters of organ function, however, may shed some light on potential determinants of irreversibility also in the brain. While liver enzyme activities in plasma normalize within weeks of abstention from drinking, other parameters such as erythrocyte mean corpuscular volume take months to years to recover (Hasselblatt et al., 2001). Regulatory hormonal circuits, e.g. the hypothalamic-pituitary-adrenal or the hypothalamic-pituitary-gonadal axis, remain impaired for months (Ehrenreich et al., 1997b; Hasselblatt et al., 2003). Low plasma urea concentrations have been reported in drinking and early abstinent alcoholics (Stamm et al., 1984; Wallerstedt and Olsson, 1978). In previous work we made the remarkable observation that the decreased plasma and urinary urea concentrations in alcoholics persist even over months and years of strictly controlled abstinence, suggesting sustained disturbances of urea synthesis within the urea cycle (Döring et al., 2003; Jahn et al., 2004).

In humans, the urea cycle is essential for the elimination of ammonia, a by-product of protein catabolism and utilization of most amino acids for gluconeogenesis. The immediate precursor of urea is arginine, which is hydrolysed to urea and ornithine by arginase. Arginine in turn is synthesized by the first four enzymes of the urea cycle, i.e. carbamoylphosphate synthetase, ornithine transcarbamoylase, argininosuccinate synthetase, and argininosuccinate. The formation of carbamoylphosphate by carbamoylphosphate synthetase and of argininosuccinate by argininosuccinate synthetase are the energy-dependent steps. The amount of arginine utilized for ureagenesis depends on arginine activity (Morris, 1992) and regulates the activation of carbamoylphosphate synthetase by N-acetyl-glutamate (Shigesada and Tatibana, 1971).

The present study was designed as a first step to uncover mechanisms underlying the persistently decreased urea levels in abstinent alcoholics. Using intravenous arginine as a substrate load for the urea cycle, the capacity of urea synthesis in vivo was challenged in abstinent alcoholics and controls. In our setting, arginine infusion, a procedure commonly and safely applied in diagnostic endocrinology (Ghigo et al., 2001), should unmask (i) a potentially altered rate of synthesis of urea cycle components, i.e. ornithine, citrulline, and argininosuccinate, (ii) a potential shift of arginine and ammonia metabolism towards alternative pathways of nitrogen removal including formation of glutamine and nitric oxide, and (iii) disturbed response patterns of glucose-regulating hormones stimulated by arginine (Merimee et al., 1965; Floyd et al., 1966).

EXPERIMENTAL SUBJECTS

After approval of the study by the Committee for Medical Ethics of the Georg-August-University, Göttingen, Germany, informed consent for participation was obtained from 15 healthy male controls and 14 male alcoholics. After inpatient detoxification of 1–2 weeks, patients joined the OLITA (Outpatient Longterm Intensive Therapy for Alcoholics) programme, where abstinence was ascertained through daily contacts (including weekends and holidays) and daily urine analyses for alcohol (Ehrenreich et al., 1997a). Median age of the subjects was 40 years (range, 34–53). Their median
body mass index (BMI) was 22.8 kg/m² (range, 18.4–29.4). Subjects had a history of 9 (median) (range, 3–24) years of alcohol dependence according to DSM IV criteria with a median daily consumption of 317 g (range, 120–960) of pure alcohol over the last half year before entering the programme. Subjects displayed no signs of liver cirrhosis as confirmed by physical and laboratory examination, and abdominal ultrasound imaging, were free of other severe or chronic illnesses and had no history of drug abuse (except for alcohol, nicotine, and caffeine). They had received clomethiazole, magnesium, potassium, and vitamin B₆ during the first week of abstinence. Starting from the second week, 50 mg oral calcium carbimide (Dipsan) was given as an aversive medication daily over the first 3 months of abstinence. From the fourth month on, patients were switched to disulfiram (Antabuse), 3 × 400 mg/week (Ehrenreich et al., 1997a). Although there is no indication from literature that acetaldehyde dehydrogenase inhibitors influence any of the parameters of interest here, this medication was stopped 2 weeks before the arginine challenge. The control group comprised 15 healthy male volunteers without evidence of alcohol or drug abuse. Controls were matched as closely as possible to patients with regard to age (39 years, range 30–59), BMI (24.9 kg/m², range 18.7–31.8), and cigarette consumption.

MATERIALS AND METHODS

Experimental procedures

After 13 weeks (range, 8–19) of controlled alcohol abstinence, the arginine infusion test was performed after an overnight fast. At 7:00 h, subjects were placed in a comfortable supine position. Two intravenous catheters were inserted in both forearms and kept open with a slow infusion of 0.9% saline for separate blood sampling and drug administration. Blood pressure and heart rate were monitored continuously. At 09:00 h, 30 g of L-arginine-hydrochloride (Braun, Melsungen, Germany) was infused intravenously over 30 min by means of an automated infusion pump (Braun, Germany). Venous blood samples were collected at time points 0 (start of infusion), 15, 30, 45, 60, 90, 120, and 180 min. Urine was collected throughout the preceding 24 h (09:00 h–9:00 h), from 09:00 h–12:00 h during the test, and for the following 24 h interval.

Analytical procedures

Plasma for determination of amino acid and hormone concentrations was collected in lithium heparin tubes, immediately separated by centrifugation (2000 g, 10 min, 4°C), and stored at –80°C until analysed. Plasma concentrations of the amino acids arginine, ornithine, citrulline, argininosuccinate, glutamine, and glutamate were measured by HPLC (Meyer et al., 1997). Except for hydroxyarginine (0.5 μmol/l), the detection limit for all amino acids was 0.8 μmol/l. Plasma concentrations of insulin, c-peptide and growth hormone were measured using commercially available immunoradiometric assays (ImmunoTech, Prague, Czech Republic). Glucagon concentrations were measured by radioimmunoassay (LINCO Research, Missouri). Glucagon-like-peptide 1 (GLP-1) concentrations were determined by enzyme-linked immunosorbent assay (ELISA; LINCO Research). Samples from patients and controls were always assayed in random order. Data on intra-assay and inter-assay variations are compiled in Table 1. Plasma and urinary concentrations of nitrate/nitrite were measured as described (Moshage et al., 1995). Blood glucose concentrations were serially determined from capillary blood (Glucometer Elite, Bayer, Germany). Total protein, albumin, ammonia, and urea were determined using standard laboratory techniques. Liver enzyme activities [alanine amino transaminase (ALAT), aspartate amino transaminase (ASAT), ammonia, and urea were determined using standard laboratory techniques. Liver enzyme activities [alanine amino transaminase (ALAT), aspartate amino transaminase (ASAT), cholinesterase (CHE), and glutaminase (GLP-1)] were determined at 37°C and were calculated equivalent to a measurement at 25°C.

Data analysis

Data are presented as mean ± SD in the text and as mean ± SEM in the figures. Statistical analysis was performed by Student’s t-test. For multiple comparisons between groups and over time ANOVA was used. Post hoc testing was done if appropriate by Duncan’s test applying the Statistica software package (Statsoft Inc., Tulsa OK). Significance level was P ≤ 0.05.

RESULTS

Baseline laboratory values

By the time the arginine stimulation test was performed, i.e. after a median of 13 weeks (range, 8–19) of strictly controlled alcohol abstinence, plasma activities of liver enzymes had normalized. Plasma concentrations of total protein and of albumin were comparable with that of controls. Notably, plasma as well as urinary urea concentrations were significantly lower in abstinent alcoholics (Table 2).

Blood pressure, heart rate, and adverse events upon arginine administration

In both abstinent alcoholics and controls mean arterial pressure (range, 85–92 mmHg) and heart rate (range, 60–75/min)
C-peptide concentrations tended to be lower in alcoholics compared to controls (19 ± 11 versus 31 ± 19 mU/l; Fig. 4C). Similarly, insulin and C-peptide concentrations increased transiently to peak at 0:30 h mirroring the insulin response to arginine infusion. Again, the increase in plasma glucagon concentrations was significantly lower in alcoholics (219 ± 60 versus 312 ± 104 pg/ml, Fig. 4D). No significant changes in plasma GLP-1 concentrations were observed over time nor between groups (data not shown).

**Growth hormone concentrations upon arginine infusion**

Plasma growth hormone concentrations increased transiently to peak at 1:00 h. In abstinent alcoholics, the increase in growth hormone concentrations was significantly augmented as compared with that in controls (1:00 h, 21.7 ± 28.9 versus 7.6 ± 11.7 mU/l) (Fig. 5).

**DISCUSSION**

The present study employed an intravenous arginine challenge to unravel persistent alterations in circulating urea cycle components and major metabolic hormones that clearly outlast the normalization of plasma liver enzyme activities in long-term abstinent alcoholics. In comparison with healthy controls, alcoholics started from lower plasma urea levels and demonstrated in response to arginine: (i) higher peak concentrations of arginine and increased arginine/ornithine and ornithine/citrulline ratios; (ii) elevated plasma glutamine levels but unaffected plasma glutamate, ammonia, and nitrate/nitrite; (iii) distinctly lower urinary urea excretion despite increases in plasma urea concentrations over baseline; (iv) a blunted plasma glucose stimulation together with a reduced insulin and glucagon surge; (v) an elevated growth hormone peak.

Although several parameters measured in plasma only indirectly reflect disturbances of *intracellular* metabolites, i.e. represent a spill-over into the extracellular space, the discovery of such a globally altered pattern of metabolic and hormonal response to arginine in strictly abstinent alcoholics may encourage further research on the mechanisms of irreversibility.

Since administration of arginine resulted in comparable increases in plasma urea over the respective baseline in both experimental groups, a reduced activity of arginase per se is unlikely to fully account for the persistently lowered urea concentrations in abstinent alcoholics. The increased arginine peak in alcoholics, in turn, could be explained by a delayed cellular uptake of arginine, which has been described in rat liver cells upon ethanol exposure (Rosa and Rubin, 1980). Similarly, a reduced capacity of the ornithine/citrulline-carrier might cause a delayed transport of ornithine into the mitochondrial compartment, contributing to an accumulation of ornithine and an increased ornithine/citrulline ratio. In any case, the elevated plasma arginine concentrations along with increased plasma arginine/ornithine and ornithine/citrulline ratios and decreased urinary urea excretion upon intravenous arginine challenge point towards an overall reduced capacity of the urea cycle in abstinent alcoholics. How else can such reduced capacity be explained?

Arginine stimulates the activation of carbamoylphosphate synthetase by *N*-acetyl-glutamate (Shigesada and Tatibana, 1971). An intravenous arginine challenge would, therefore,
Fig. 1. Plasma concentrations of urea cycle metabolites and corresponding ratios following intravenous arginine challenge (30 g) in abstinent alcoholics (n = 14, closed circles) and healthy controls (n = 15, open circles) over time (h:min). *P < 0.05, **P < 0.01.
Fig. 2. Plasma concentrations of glutamate (A), glutamine (B), and nitrate/nitrite (C) upon arginine administration in abstinent alcoholics (n = 14, closed circles) and healthy controls (n = 15, open circles) over time (h:min). *P < 0.05, **P < 0.01.

Fig. 3. Amount of urinary urea (A) and nitrate (B) in abstinent alcoholics (n = 14, black bars) and healthy controls (n = 15, white bars) before, during and after intravenous arginine load. *P < 0.05, **P < 0.01.

Fig. 4. Capillary blood concentrations of glucose (A), and plasma concentrations of glucose-regulating hormones, insulin (B), C-peptide (C), and glucagon (D), upon arginine administration in abstinent alcoholics (n = 14, closed circles) and healthy controls (n = 15, open circles) over time (h:min). *P < 0.05, **P < 0.01.
be expected to increase the capacity of the urea cycle, resulting in enhanced turnover and, subsequently, rapid normalization of plasma arginine and ornithine concentrations. The finding of increased plasma arginine concentrations together with increased plasma arginine/ornithine and ornithine/citrulline ratios in abstinent alcoholics indirectly indicates a decreased activity of carbamoylphosphate synthetase. Since the generation of carbamoylphosphate is the first ATP-dependent step within the urea cycle, such decreased activity of carbamoylphosphate synthetase might best be explained by a reduced availability of ATP. Indeed, chronic ethanol exposure has been shown to result in depression of hepatic mitochondrial energy metabolism causing impaired citrulline and urea synthesis in rat hepatocyte mitochondria upon ethanol exposure (Cunningham et al., 1990; Adachi et al., 1995).

Accordingly, our finding of significantly higher glutamine plasma concentrations in abstinent alcoholics following the arginine challenge might reveal a compensatory shift towards elimination of ammonia by glutamine synthetase, as has been reported for chronic ethanol exposure in perivenous rat hepatocytes (Garcia-Ruiz et al., 1994). Exhaustion of this reserve under pathophysiological conditions may predispose alcoholics, even after long-term abstinence, for cerebrotoxic consequences due to both elevated glutamine and ammonia levels (Cooper, 2001). The latter were found to be unaltered in our experimental setting, pointing to a still sufficient elimination of ammonia. In addition, findings of unchanged plasma concentrations of nitrite/nitrate and hydroxynitrin in response to arginine argue against an increased conversion of arginine to nitric oxide in abstinent alcoholics. Regarding the higher plasma glutamine levels in abstinent alcoholics after arginine infusion, cortisol driven glutamine synthesis and release cannot be entirely ruled out as a contributing mechanism.

Under fasting conditions, arginine is utilized for the generation of glucose. The blunted glycaemic response of alcoholics to intravenous arginine may point (along with the significantly lower insulin and glucagon peaks) towards impaired gluconeogenesis. Impaired gluconeogenesis, in turn, further reflects the long-term disturbance of energy metabolism in abstinent alcoholics. Indeed, our findings in alcoholics closely resemble experimental data on the pharmacological blockade of gluconeogenesis (Trabelsi et al., 1995). As described previously for healthy controls (Herrmann et al., 1995), intravenous arginine did not elicit changes in plasma GLP-1 concentrations in abstinent alcoholics.

In contrast to the blunted insulin and glucagon peaks, the growth hormone response to arginine was augmented in alcoholics. Although the cause of this dissociating response is still unclear, it may partly explain the reduced urea production/excretion in alcoholics after arginine load. In fact, the opposite constellation, growth hormone deficiency (Palekar et al., 1982; Dahms et al., 1989) as well as high glucagon (Snodgrass et al., 1978) are known to stimulate the generation of urea.

The persistently decreased plasma and urinary urea concentrations in abstinent alcoholic men may not only be due to an impaired energy metabolism and an imbalance of glucose-regulating hormones, as delineated above, but also to persistent disturbances of hormonal regulatory systems of electrolyte and water balance (Döring et al., 2003). In fact, we have previously shown lasting suppression of circulating vasopressin in long-term abstinent alcoholics (Döring et al., 2003). Vasopressin increases urea synthesis in isolated rat hepatocytes by enhancing the rate of mitochondrial citrulline synthesis (Corvera and Garcia-Sainz, 1982) and stimulates glutaminase activity (Corvera and Garcia-Sainz, 1982; Drew et al., 1985).

To conclude, application of an intravenous arginine challenge uncovers persistent metabolic and hormonal disturbances in abstinent alcoholics, affecting the urea cycle and gluconeogenesis. The common denominator of many of these changes appears to be an acquired irreversible deficiency in cellular energy regulation. This deficiency may predispose alcohol-dependent men, despite long-term abstinence, for a more rapid decompensation of metabolic/protective cellular pathways. Similar mechanisms in the brain may underlie the phenomenon of dependence.

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REFERENCES


