ADENYLYL CYCLASE ACTIVITY IS INCREASED IN YOUNGER, BUT DECREASED IN OLDER, ALCOHOLIC PATIENTS AFTER DETOXIFICATION

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Abstract — Acute and chronic administration of ethanol leads to alterations of the adenylyl cyclase (AC) signal transduction pathway. This study examined whether the formation of cAMP by AC in lymphocytes correlates with age in alcoholic patients and in healthy controls. Blood was drawn for preparation of lymphocyte membranes and for determination of basal, GTPγS-stimulated, and forskolin-stimulated AC activity from 68 actively drinking alcoholic patients (age, mean ± SD: 45 ± 10; range: 26–69 years) after ethanol detoxification. The patients’ AC activity correlated negatively with age. In contrast, no effect of age was observed in the healthy controls (age, mean ± SD: 42 ± 11; range: 24–65 years). The age-related decrease in AC activity of alcoholic patients could not be attributed to the duration of regular alcohol intake. It was partly due to the large variance of AC activity in younger and middle-aged alcoholics.

INTRODUCTION

Ethanol influences the activity of several signal transduction systems at the receptor level as well as at the postreceptor level (Torres and Horowitz, 1996). One target is the adenylyl cyclase (AC) system (Rabin and Molinoff, 1983). AC is activated via a receptor modulated G-protein and generates cAMP, which becomes degraded by phosphodiesterases. The second messenger cAMP activates the phosphorylating enzyme protein kinase A (for review see Sunahara et al., 1996). In most paradigms, acute ethanol administered in clinically relevant doses leads to an increase in AC activity, whereas chronic ethanol administration is associated with a down-regulation in AC activity (Diamond et al., 1987; Mochly-Rosen et al., 1988; May et al., 1995; Yoshimura and Tabakoff, 1995). Several clinical studies have raised the possibility that the AC activity of alcohol-dependent patients differs from that of normal controls (Diamond et al., 1987; Nagy et al., 1988; Waltman et al., 1993; Wand et al., 1994; Parsian et al., 1996). Consequently, measurements of AC activity in peripheral blood cells have been proposed as a means of identifying alcohol-dependent persons or persons at increased risk of becoming alcohol dependent (Diamond et al., 1987). One potentially confounding factor in such studies is the age of the persons under study. Changes in the responsiveness and the basal activity of the receptor/G-protein/AC signal transduction cascade system are considered part of the ageing process in animals and humans. The declination of heart muscle cells to β-adrenergic stimuli, the declining ability of kidneys to concentrate urine following exposure to antidiuretic hormone, or the age-related downregulation of the AC system in various brain areas including the striatum are examples of the influence of age on the function of the AC system (Sugawa and May, 1993; Davidson et al., 1995; Brodde et al., 1995; Scarpace et al., 1996). Although it appears to be the case more often than vice versa, age is not always associated with AC downregulation. For example, Graham et al. (1987) observed that β-receptor-mediated hepatic glycogenolysis in the aged rat is predicated upon increases in the density of β-receptors as well as increased intrinsic activity of the catalytic subunit of AC. Parenti et al. (1991) described an almost threefold increase in AC activity from pituitary membranes from aged rats, compared to young and adult rats.

Our study aimed to evaluate the correlation of age and basal and stimulated AC activity of lymphocytes from alcohol-dependent patients after detoxification in comparison to healthy control persons.

MATERIALS AND METHODS

Patients and controls

Sixty-eight actively drinking male patients admitted consecutively for detoxification to the Department of Psychiatry of the University of Mainz, Germany suffering from alcohol dependence according to the criteria of DSM-IV (American Psychiatric Association, 1994) were included in this study (mean age ± SD: 45 ± 10 years; range: 26–69 years) after informed written consent was obtained. The study was approved by the local ethics committee. Main exclusion criteria were abuse of psychoactive drugs other than ethanol or cigarette smoking, as well as a history of bipolar affective disorder or schizophrenia. Peripheral blood was drawn 10 to 14 days later at the time of discharge. Because of the suspected effects of posture on activity of AC lymphocytes (Mader et al., 1988), blood was drawn in the supine or sitting position. Patients were treated with different doses of clomethiazol (Distraneurin®), depending on the severity of alcohol-withdrawal symptoms. Patients were age-matched with non-alcoholic healthy male controls (n = 44; mean age: 42 ± 11 years; range: 24–65 years). None of the patients or the controls received benzodiazepines at the time of study.

Preparation of lymphocytes

Lymphocytes were isolated by centrifugation through a Ficoll gradient (Boyum, 1968). Peripheral blood was collected in 9-ml EDTA tubes (1.6 mg/ml blood; Sarstedt, Germany) and diluted 1:3 with 1 x PBS buffer (phosphate-buffered
Preparation of lymphocyte membranes

As described previously (Pauly et al., 1999), lymphocytes were thawed and resuspended to a concentration of 10^7 cells/ml in buffer I, 10 mM HEPES (ICN Biochemicals, Meckenheim, Germany), 2 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N′,N″,N‴-tetraacetic acid; ICN Biochemicals), 4 mM MgCl₂ (Fluka, Neu-Ulm, Germany), pH 6.8. Ninety-five to 98% of the lymphocytes were disrupted by 60 strokes (glass/glass; type S) in a precooled Dounce homogenizer (Braun, Melsungen, Germany). Nuclei were separated by 92-min centrifugation at 500 g and 4°C. Membranes were pelleted by a 30-min centrifugation at 4500 g and 4°C. The membrane pellet was resuspended in 200 µl of buffer II, containing 10 mM Tris/HC1 pH 7.4 (Roth, Karlsruhe, Germany), 1 mM EDTA (Sigma, Munich, Germany), 0.5 mM DTT (dithiothreitol; Sigma), 0.5 mM PMSF (phenylmethylsulphonylfluoride; Fluka), 1 mM benzamidine (Sigma), 0.1 mM benzethonium-chloride (Sigma), 0.5 µM sodiumorthovanadate (Na₃VO₄; Sigma), 64 ng STI (soybean trypsin inhibitor; Sigma), 1.76 µg TPCK (L-1-chlor-3-(4-tosylamido)-4-phenyl-2 butanon; Boehringer Mannheim, Germany), 440 ng TLCK (Nα-tosyl-L-lysine-chloromethyl ketone hydrochloride; Boehringer Mannheim), 56 ng aprtinin (from bovine lung; Trasylol; Fluka), 12 mM Tris, 2 mM MgCl₂, 10% glycerol (87%; Fluka). An aliquot was taken for determination of membrane protein content (Bradford, 1976) using bovine serum albumin as standard (Protein-assay; Bio-Rad, Munich, Germany). The membrane fraction was frozen at −80°C for a minimum of 12 h until further use. Storage of more than 6 months had no measurable influence on enzyme activity.

AC assay

As a measure of AC activity, the formation of ^32^P-cAMP from α^32^P-ATP was quantified. Frozen membranes were thawed and resuspended for 30 min at 41 000 g and 4°C. Membranes were diluted with buffer III, pH 6.8, containing 20 mM HEPES, 3 mM EGTA, 0.53 mM CaCl₂ (Fluka), 3 mM MgCl₂, 110 mM KCl (Sigma), 10 mM LiCl (Sigma), 0.1 mM ATP (ICN Biochemicals) to a final concentration of 1 µg protein/µl. Aliquots of 20 µl were then incubated for 10 min at 30°C with 100 µl of reaction mixture containing 30 mM MOPS (Roth), 15 mM creatine phosphate (ICN Biochemicals), 4.5 mM theophylline (Sigma), 16 mM ATP, 0.5 mM DTT, 5 mM MgCl₂, 50 µg creatine phosphokinase (Sigma), 0.45–0.5 µCi α^32^P-ATP (specific activity: 3000 Ci/mmol; NEN-Dupont, Wilmington, DE, USA). For stimulation, the reaction mixture contained additionally either 20 µM guanosine-5′-o-(3-triphosphate) (GTPγS; Sigma) or 100 µM forskolin (Sigma). The reaction was stopped by adding 500 µl of Stop-mix consisting of 2% SDS (Roth), 0.1 mM cAMP (Fluka), and ^3^H-cAMP (specific activity: 250 mCi; NEN-Dupont) which served as internal standard.

Separation of substrate and reaction product was achieved by column chromatography on Al₂O₃ material (aluminium oxide 90 active, neutral; Merck, Darmstadt, Germany). Columns (internal diameter:1 cm) were filled with a slurry of 2.5 ml Al₂O₃ in 1:1.5 w/v 0.1 M imidazole pH 7.4 (Sigma) and rinsed with 2 ml of 0.1 M imidazole pH 7.4 prior to use. The volume of the reaction mixture was adjusted to 1 ml with 0.1 M imidazole pH 7.4 (Sigma) and applied to the column.

The first elution was carried out by applying 1 additional ml of 0.1 M imidazole, pH 7.4, to the column and collecting the eluent (2 ml) in a 6-ml scintillation vial (Beckman Instruments, Munich, Germany). Thereafter, the elution was repeated three times, each time applying 2 ml of 0.1 M imidazole, pH 7.4, to the column and collecting the eluent in separate scintillation vials. Eluent samples were mixed with 2 ml of a scintillation cocktail (Ready-Gel, Beckman Instruments) and measured by liquid scintillation spectroscopy using the LS 6000 series liquid scintillation system (Beckman Instruments). The counts per minute value of the first elution containing background radiation was subtracted from the counts per minute values of the eluent samples containing the ^3^H-labelled internal standard and thus the ^32^P-cAMP product.

Statistical analysis

For evaluation of correlations between age and AC activity, the correlation coefficient was calculated using the Spearman rank correlation coefficient. Correlations were considered as significant for P < 0.05. The two-tailed t-test was used in the evaluation of group differences. Differences were considered as significant for P < 0.05. The Levene test for the equality of variances was used.

RESULTS

In alcoholic patients, basal AC activity, GTPγS-stimulated AC activity, and forskolin-stimulated AC activity showed a marked reduction with increasing age. The respective correlation coefficients were −0.27, −0.34, and −0.35 with the associated P-values of 0.026, 0.003, and 0.005 (Fig. 1). When patients were grouped into three age groups, under 40 years (n = 27; controls: n = 21), between 41 and 54 years (n = 29; controls: n = 15), and over 55 years (n = 12; controls: n = 8), the mean AC activity of the younger groups consistently exceeded that of the older groups. Differences were highly significant for the comparison between the extreme age groups (Table 1). The observed differences could partly be attributed to the large variance in AC activity in younger and middle-aged alcoholics. Variances in AC activity were significantly larger in the alcoholic group than in the control group. The P-values of the Levene test for equality of variances were 0.086, 0.037, and 0.043 for basal, GTPγS-stimulated, and forskolin-stimulated AC activity respectively.

In contrast, in the healthy controls, no significant age-related effects could be demonstrated, AC activity was age-invariant (Fig. 1). Correlation coefficients were −0.08, −0.07, and −0.25 with associated P-values of 0.6, 0.7, and 0.09.
When patients’ mean AC activity ($n = 68$) was compared with those of controls ($n = 44$), patients showed a trend towards increased AC activity under all conditions tested, which, however, did not consistently reach significance levels (Table 1). In particular, mean AC activity in patients under 40 years of age was higher than that of the corresponding controls. The respective $P$-values were 0.02, 0.01, and 0.02 for basal, GTP$\gamma$S-stimulated, and forskolin-stimulated AC activity. The AC activity of patients in the over 55-year age group was significantly lower than those of the controls with respective $P$-values of 0.020, 0.004, and 0.01.

To find out whether the observed age-related reductions of enzyme activity in the alcohol-dependent patients were also related to the duration of regular alcohol intake, we additionally correlated basal and stimulated AC activity with the duration of regular alcohol intake as indicated by the respective item (years of ethanol consumption on three or more occasions per week, item 1b of the drug and ethanol use section) of the Addiction Severity Index (ASI, 5th edn, McLellan et al., 1992) that was available for 44 patients. Under these conditions, no significant effect of the duration of ethanol intake on AC activity could be demonstrated (Fig. 2).
In this study, we found that, in alcoholic patients, the stimulated and basal AC activity of peripheral lymphocytes are negatively correlated with age. The negative correlation was significant for basal AC activity as well as for enzyme activity after stimulation with the GTP-analogue GTPyS and the diterpene forskolin. In healthy controls, AC activity was not significantly different between young and older subjects. In addition, no correlations were found for lymphocyte AC activity and the duration of regular ethanol intake, although the duration of ethanol intake correlated positively with the age of the patients. This finding might be explained by the assumption that the duration of ethanol intake does not adequately reflect the cumulative amount of ethanol consumed, which might be the more relevant factor. However, the amount of ethanol consumed is notoriously hard to measure in alcohol-dependent patients and was not quantified in this study. Alternatively, the decrease in AC activity in aged, detoxified alcoholic patients might be a function of the dependence, rather than of the ethanol consumption.

Alcoholic patients differ in many respects from healthy controls, so that group differences cannot simply be attributed to the ethanol consumption nor to dependence, but may also be influenced by different life styles, or dietary and other factors. In particular, cigarette smoking is more frequent among alcoholic patients, than healthy controls. Because lymphocytes express nicotinic acetylcholine receptor channels (Hiemke et al., 1996), varying smoking habits might account for some of the differences. However, these channels are not directly coupled through the AC system and no systematic effects on AC activity were found when smokers and non-smokers were compared within each group. In addition, published experimental data do not support the hypothesis that nicotine has a major impact on AC activity in peripheral lymphocytes (Geng et al., 1996).

When mean AC activity of the patients and controls were compared, the patients showed a trend towards increased AC activity that was largely due to the large variance in younger and middle-aged alcoholics. The increased variance of AC activity in the alcoholic patients deserves attention, because it appears to reflect a distortion of the AC signalling system in these patients that may be related to alcoholism. So far, we cannot offer a conclusive explanation why some patients showed AC activity far different from the mean activity. As we have pointed out in previous studies (Szegedi et al., 1998; Pauly et al., 1999), actual blood-ethanol levels of actively drinking patients as well as acute withdrawal effects may modulate basal and stimulated AC activity in peripheral blood cells. Because we measured activity only days after the cessation of withdrawal symptoms, state-dependent factors may still play a role.

Several studies have reported a difference in AC activity in blood cells between alcoholic patients and controls, but the influence of age has not previously been systematically studied. Diamond et al. (1987) showed that lymphocytes of 10 actively drinking patients (mean age 48.6 ± 3.6 years) exhibited reduced basal and adenosine receptor-stimulated AC activity of lymphocytes from alcoholic patients after ethanol detoxification (n = 44) in relation to the duration of regular ethanol intake.

Fig. 2. Adenyl cyclase (AC) activity of lymphocytes from alcoholic patients after ethanol detoxification (n = 44) in relation to the duration of regular ethanol intake.

Duration group I: 0–9 years of regular consumption (n = 9); group II: 10–19 years (n = 13); group III: 20–29 years (n = 15); group IV: 30–40 years (n = 7). Shown are basal activity (Basal), activity after stimulation with 20 µM GTPyS (GTPyS) and activity after stimulation with 100 µM forskolin (FORS). AC activity of the detoxified alcoholic patients was not correlated with the duration of regular ethanol consumption.

Nevertheless, the duration of regular ethanol intake was correlated with the age of the patients (correlation coefficient 0.43; P = 0.002). No systematic effects on AC activity were found, when smokers and non-smokers were compared within each group (data not shown).

**DISCUSSION**

In this study, we found that, in alcoholic patients, the stimulated and basal AC activity of peripheral lymphocytes are negatively correlated with age. The negative correlation was significant for basal AC activity as well as for enzyme activity after stimulation with the GTP-analogue GTPyS and the diterpene forskolin. In healthy controls, AC activity was not significantly different between young and older subjects. In addition, no correlations were found for lymphocyte AC activity and the duration of regular ethanol intake, although the duration of ethanol intake correlated positively with the age of the patients. This finding might be explained by the

| Table 1. Adenyl cyclase (AC) activity in patients and controls in different age groups |
|----------------------------------------|-----------------|-----------------|-----------------|
| Age group (years) | Patients’ AC | Controls’ AC |
| | Basal | GTPyS | FORS | Basal | GTPyS | FORS |
| <40 | 2320<sup>a</sup> | 6200<sup>a</sup> | 14 490<sup>a</sup> | 1380 | 3620 | 10 340 |
| ±1920 | ±4650 | ±8200 | ±320 | ±1060 | ±2130 |
| 41–54 | 2150 | 4860 | 12 130 | 1290 | 3410 | 9790 |
| ±1210 | ±2740 | ±6450 | ±410 | ±1120 | ±2300 |
| >55 | 950<sup>c</sup> | 2430<sup>c</sup> | 6600<sup>c</sup> | 1270 | 3810 | 8950 |
| ±380 | ±1470 | ±3490 | ±280 | ±950 | ±1690 |
| All | 2010 | 4960 | 12 090 | 1330 | 3580 | 9900 |
| ±1520 | ±3690 | ±7290 | ±340 | ±1050 | ±2140 |

Values are given in pmol cAMP/mg protein × min and are means ± SD. Significance (t-test, two-sided) is indicated as follows: <sup>a</sup>P < 0.05 vs controls < 40 years; <sup>b</sup>P < 0.0001 vs patients < 40 years; <sup>c</sup>P < 0.05 vs controls > 55 years. GTPyS, FORS: AC activity after stimulation with GTPyS or forskolin, respectively.
activity, compared to non-alcoholic controls. Parsian et al. (1996) described significantly reduced basal and caesium fluoride-stimulated AC activity in platelets of 51 male alcoholic patients, compared to 54 normal controls. However, the mean age of the patients was only 31.7 ± 10.9 years. Furthermore, blood-ethanol levels, which were not specified in the aforementioned studies, might also be responsible for the reported changes in the AC system. This point is also made in the study by Waltman et al. (1993), in which AC activity in lymphocytes of 22 actively drinking alcoholic patients was compared with that of 41 abstinence alcoholics and 54 controls. The AC activity was reduced in the (abstinence) alcoholics, but non-significantly increased in the actively drinking alcoholic patients.

The finding that age did not affect AC activity of lymphocytes in healthy controls might be perceived as surprising, since a decline in the responsiveness of the AC system is part of the ageing process in many tissues. However, the age dependency of AC activity of human lymphocytes has not as yet been studied conclusively, and conflicting data have been reported in the literature. Mader et al. (1988), in agreement with our data on healthy controls, were unable to demonstrate any age-related differences in basal, isoproterenol- or forskolin-stimulated AC activity. In contrast, Krall et al. (1981) reported an age-related reduction in lymphocyte AC activity. Likewise, relatively little is known about the effects of ageing on related components of the signal transduction machinery. Barki-Harrington et al. (1996) found the G-proteins Ga15, Gs, and Gi1 in lymphocytes did not change with age. Total lymphocyte activity of protein kinase C, a kinase only indirectly affected by cAMP by cross talk mechanisms, has been described as inversely related to age (De Petriolo, 1994).

Since the age-dependent decrease was found for both basal and stimulated AC activity, we conclude that the observed decline was most probably attributable to the AC protein, rather than to other parts of the second messenger system. It remains to be shown how far the observed changes were also relevant for receptor-mediated stimulation of AC. Based on our findings, it seems likely that stimulation of a G-protein coupled receptor would similarly decrease with age in alcoholic patients.

In summary, this study provides evidence that the AC activity of lymphocytes of detoxified alcoholic patients is inversely correlated with the age of the patients, but not with the duration of regular alcohol intake. Decreased AC activity was present in patients older than 55 years, whereas younger patients showed a greater variance than controls.

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