Biological vulnerability to alcoholism in children of alcoholics
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Platelet adenylyl cyclase activity as a biochemical trait marker for predisposition to alcoholism*

ABSTRACT

Previous studies demonstrated a reduced $G_s$-protein stimulated adenylyl cyclase activity in the brain and blood cells of alcoholics. We investigated this phenomenon in platelets of children of alcoholics (COAs), i.e., of children at high risk for the acquisition of alcoholism and (as yet) not regularly consuming alcohol. $G_s$-protein mediated stimulation of adenylyl cyclase by 30 mM NaF and 50 iM forskolin stimulated adenylyl cyclase activity were assessed in platelet membranes of 23 (male and female) COAs and 20 control children. $G_s$-protein stimulated cAMP production by NaF, unlike that induced by direct stimulation of adenylyl cyclase with forskolin, in platelet membranes of COAs was profoundly lower than in platelet membranes of control children. Moreover, such a reduced $G_s$-protein functioning was only observed in platelet membranes of COAs with a multigenerational family history of alcoholism. A reduction of $G_s$-protein stimulated adenylyl cyclase activity in platelets may represent a sensitive and gender-independent trait marker for predisposition to alcoholism, rather than a state marker for alcoholism.

INTRODUCTION

Alcoholism is a widely occurring psychiatric syndrome in our Western society for which as yet no clearly effective pharmacotherapy is available. Because a genetic contribution for the development of alcoholism was found in behavioral genetic studies,\textsuperscript{1,2} it may be possible to identify a biological diagnostic trait marker indicating enhanced vulnerability to alcoholism. Such a trait marker has to be genetically transmitted, co-segregated with alcoholism in affected relatives, and particularly more prevalent in individuals at high risk than in the general population. Moreover, the marker must be stable over time, present during remission of alcoholism, and easily and reliably detectable.\textsuperscript{3} In this respect, a variety of possible electrophysiological and biochemical traits have been evaluated.\textsuperscript{4}

So far, the most promising biochemical trait marker seems to be a reduced guanine nucleotide binding (G\textsubscript{s}) protein stimulated adenylyl cyclase activity in platelets and lymphocytes\textsuperscript{5-8}, as found in the brain of alcoholics.\textsuperscript{9} Unfortunately, a major shortcoming of these studies is that the observed difference in platelet adenylyl cyclase activity between the adult alcoholics and controls may well be due to persistent changes in blood cells as a consequence of high daily alcohol consumption. Before accepting platelet or lymphocyte adenylyl cyclase activity as a reliable trait marker, it is crucial to show its altered activity in alcohol-naive persons with a high risk to develop alcoholism, such as children of families with multi-generational alcoholism.\textsuperscript{10} Devor et al\textsuperscript{11}, studying adenylyl cyclase activity in platelets, observed that fluoride-stimulated activity of the enzyme displays a single major locus effect, suggesting a possible Mendelian genetic transmission.
Therefore, children of alcoholics (COAs) that do not regularly consume alcohol may represent an excellent population to investigate whether or not adenylyl cyclase activity represents a trait marker for predisposition to alcoholism rather than a state marker for alcoholism. Therefore, we compared fluoride and forskolin stimulated adenylyl cyclase activity in platelets of COAs from families with multi-generational alcoholism, with that of COAs from families with first degree alcoholism and control children.

METHODS

*Experimental Groups*

This study included 43 children: 23 children of 17 parents (3 mothers, 14 fathers) treated for alcoholism in addiction centers in the Amsterdam region and a control group of 20 children of 20 parents. All children were Caucasian, except for two control children (1 Turkish child, 1 Black child). The age of the 23 children (9 boys, 14 girls) ranged from 8-18 years (mean age 12.5 ± 0.7 years). The age of the 20 control children (7 boys, 13 girls) ranged from 6-17 years (mean age 12.0 ± 0.8 years). There was no difference between the two groups on a three points socio-economic status (SES) score (mean score for COAs, 2.2 ± 0.2; control children, 2.2 ± 0.2). The study was approved by the Medical Ethical Committee of the Academic Medical Center (AMC). Parents and children 12 years old and older gave their written, informed consent.
Alcoholism in the 17 parents, comprised alcohol abuse \((n = 1)\) and alcohol dependence \((n = 16)\), as assessed using DSM-III-R criteria with the Composite International Diagnostic Interview (CIDI).\(^{12}\) An antisocial personality disorder was assessed in the parents of the COAs according to DSM-III-R criteria. In only one parent was an antisocial personality disorder present. The COAs were screened for the presence of morphological anomalies representing fetal alcohol effects.\(^{13}\) COAs with fetal alcohol effects or probable fetal alcohol effects were not included in the study. Mothers of the COAs were asked how many standard drinks they consumed daily during their pregnancy: three mothers reported to drink zero to one standard drink daily; one mother reported to drink one to two standard drink daily; all the other mothers reported that they have not been drinking during their pregnancy. Of all 43 children, problem behavior, alcohol use, and frequency of smoking in the last 4 weeks were assessed with the Child Behavior Checklist (CBCL)\(^{14}\) and a substance use questionnaire (NIAD list, Trimbosinstituut Utrecht, The Netherlands). None of the children had to be excluded because of regular alcohol consumption (i.e., more than two standard drinks in one week during the last month) or drug abuse or dependence. Alcoholism in first- and second-degree family members was assessed by the Family History Section (FHS) of the EuropASI.\(^{15}\) We used the criteria according to Dawson et al\(^{16}\) to make a comparison among the three groups of children: (1) COAs from families with multi-generational alcoholism, with alcoholism in first- and second-degree biological relatives (the family-history positive, FHP-multi group), (2) COAs from families with first degree alcoholism, with alcoholism in first- but not in second-degree biological relatives (the FHP-first-degree group) and (3) control children with neither-group alcoholism in parents nor in siblings.
The 20 control children were healthy outpatients: one group of 8 children visited the AMC pediatric outpatient department for a medication check-up (6 children were stable using physiological supplements of thyrax and 2 children were stable on physiological supplements of cortisone), the other group of 12 children had otorhinolaryngological or dental surgery. As expected no differences in outcome values of basal levels, forskolin or fluoride stimulated adenylate cyclase activity were found between these two subgroups of controls. The age slightly differed (mean 13.0 vs. 10.5 years). Twelve of the 23 COAs had a multi-generational family history of alcoholism and 11 COAs had a first-degree family history of alcoholism. Medication for allergic asthma was used by one FHP-multi COA (salmeterol, salbutamol and beclometasone) and one FHP-first-degree COA (cromoglicic acid); no other medications were used by the COAs. In both groups of COAs, but not in the control group, four children suffered from allergic asthma. No influence of allergic asthma was found on all outcome levels. The age of the 12 FHP-multi COAs (5 boys, 7 girls) ranged from 8-18 years (mean age 12.7 ± 1.1 years). The age of the FHP-first-degree COAs (4 boys, 7 girls) ranged from 9-17 years (mean age 12.4 ± 0.9 years). The SES score showed no difference between FHP-multi COAs and FHP-first-degree COAs in (mean score FHP-multi COAs, 2.2 ± 0.2; FHP-first-degree COAs, 2.3 ± 0.2). In the whole group of children (COAs + controls) and in the COA group (FHP-multi + FHP-first-degree COAs), we assessed the possible influence of age and sex, SES, storage time of frozen platelets, CBCL total problem T-score, frequency of alcohol use, and frequency of smoking of the NIAAD list on all outcome levels to find possible confounding effects.
Adenylyl Cyclase Assay

Blood (14 ml) from all 43 children was taken by venipuncture and immediately centrifuged (180 x g for 10 min. at 4°C). The plasma was centrifuged at 132 x g for 10 min. at 4°C to form 2-3 ml platelet rich plasma (PRP). 0.5 ml PRP was used to count the platelets. Platelets were subsequently sedimented by centrifugation at 10,000 x g (10 min. at 4°C). The pellet was stored at -70°C. The pellets were thawed and resuspended to form platelet membranes in 5 ml of 150 mM NaCl, 20 mM EDTA, and 50 mM Tris-HCl, pH 7.4, using a sonicator. The material was centrifuged at 38,000 x g for 10 min. at 4°C, and the resultant pellet was resuspended in 5 ml of 5 mM Tris-HCl and 5 mM EDTA, pH 7.4, using a hand homogenizer. The suspension was centrifuged at 38,000 x g for 10 min. at 4°C, and the pellet was resuspended in 1 ml of 5 mM Tris-HCl and 1 mM EDTA, pH 7.4, using a hand homogenizer. This suspension of platelet membranes was used for assays of adenylyl cyclase activity.

To determine adenylyl cyclase activity in the samples, the reactions were initiated by adding 50 µl of platelet membranes (protein concentrations 0.03-0.43 mg/ml) in the reaction mixture (100 µl) and incubated for 10 min. at 30°C, without (basal activity) or with (stimulated activity) 50 µM forskolin or 30 mM NaF. The reaction mixture (100 µl) consisted of 50 mM Tris-HCl, 10 mM MgCl₂, 0.5 mM ATP, 1 mM theophylline, 10 mM phosphocreatine and 50 U/ml creatin e kinase. To stop the reaction, the samples were kept at 90°C for 3 minutes. The total amount of cAMP (pmol/mg protein/min) produced during the reaction was assessed using the protein binding method of Norsted and Fredholm. With this method, the amount of [³H]cAMP, bound in competition with experimentally produced cAMP to protein kinase
A, is determined by filtration. The radioactivity on the filters was determined by liquid scintillation counting. In each sample, basal and stimulated cAMP production was determined in triplicate.

**Statistics**

In order to reduce variability (between assays), 2-3 COA and 2-3 control samples were simultaneously determined in 8 separate experiments. The NaF and forskolin stimulated cAMP production (in excess of basal cAMP production) in the platelets of COAs was expressed as percentage of values obtained in the platelets of control children.

Skewness and kurtosis values were assessed to ascertain whether these stimulated levels were normally distributed. With this normal distribution, statistical significance of the differences between the NaF stimulated cAMP formation in COA and the control samples was assessed with the Student’s $t$ test.

Additionally, multiple regression analyses were used to study the influence of the confounders age, sex, SES, storage freezing time of platelets, CBCL total problem T-score, frequency of alcohol use score, and smoking on NaF and forskolin stimulated cAMP levels.

**RESULTS**

*Fluoride- and Forskolin-Stimulated Adenylyl Cyclase*

Preliminary experiments revealed that fluoride and forskolin caused a concentration-dependent increase in platelet cAMP production with a maximal effect at 30 mM NaF and 50 $\mu$M forskolin (data not shown). Therefore, these drug concentrations were used in the present experiments. Control and COA samples (2-3 per experiment) were simultaneously run in
each experiment for a reliable comparison of platelet adenylyl cyclase activity, given between assays variability in cAMP production.

The basal cAMP production in platelets from control children \((n = 20)\) amounted to 0.29 ± 0.06 pmol/mg protein/min and in platelets from COAs \((n = 23)\) to 0.25 ± 0.04 pmol/mg protein/min. In control samples 30 mM NaF and 50 μM forskolin induced an approximately 6- and 34-fold increase in cAMP production. The NaF- and forskolin-stimulated cAMP production, in excess of basal cAMP production, amounted to 1.71 ± 0.43 and 9.82 ± 1.88 pmol/mg protein/min, respectively. As shown in Fig.1 NaF-stimulated cAMP production in COA platelets \((1.10 ± 0.24 \text{ pmol/mg protein/min})\) was about 30% lower \((t = 2.20, df= 41, p = 0.03)\) than that in control platelets. Platelets obtained from FHP-multi COAs had a 50% \((t = 3.56, df= 21, p = 0.002)\) lower fluoride-stimulated cAMP production \((0.82 ± 0.18 \text{ pmol/mg protein/min})\) than platelets from FHP-first-degree COAs \((1.43 ± 0.46 \text{ pmol/mg protein/min})\). Interestingly, fluoride-stimulated adenylyl cyclase activity in platelets of the FHP-first-degree COAs was similar to that in platelets of control children (Fig.1). Forskolin-stimulated cAMP production was similar in the two experimental groups, COAs \((9.78 ± 1.58 \text{ pmol/mg protein/min})\) and controls (see above) (Fig.2). Forskolin-stimulated adenylyl cyclase activity did not differ between platelets of FHP-first-degree \((9.96 ± 3.28 \text{ pmol/mg protein/min})\) and FHP-multi COAs \((9.63 ± 1.21 \text{ pmol/mg protein/min})\) (Fig.2).
Fig. 1. NaF-stimulated cAMP production in platelets of COAs (n = 23), controls (n = 20), FHP-multi COAs (n = 12) and FHP-first-degree COAs (n = 11) expressed as percentage of controls (n = 20). *p < 0.05, **p < 0.005.

Fig. 2. Forskolin-stimulated cAMP production in platelets of COAs (n = 23), controls (n = 20), FHP-multi COAs (n = 12) and FHP-first-degree COAs (n = 11) expressed as percentage of controls (n = 20).
Possible Confounding Factors

Additional analysis of possible confounding effects in the whole group (COAs + controls) showed an effect of SES \([F(1,37) = 5.69, p = 0.022]\) on fluoride-stimulated cAMP production (children with a lower SES had a lower cAMP production). This was independent of, and additional to, the COA-control status and independent of FHP-multi or FHP-first-degree status of the COAs. No differences existed in SES score between the four groups, as was mentioned previously (see method section). The variables age, sex, storage time of frozen platelets, CBCL total problem T-score, frequency of alcohol use, and frequency of smoking showed no relationship with fluoride stimulated adenylyl cyclase activity. In the COA group, we found no significant effect of the confounding variables on the fluoride stimulated adenylyl cyclase activity. Analysis of the confounding effects on forskolin stimulated cAMP production showed a significant effect of sex in the whole group \([COAs + controls: F(1,37) = 8.70, p = 0.0055]\) as well as in the COA group separately \([F(1,21) = 9.80, p = 0.0051]\); girls having a lower cAMP production than boys. This sex effect did not depend on COA-control status or family history of alcoholism. The other confounding variables had no effect on the outcome variable of forskolin stimulated adenylyl cyclase activity.

DISCUSSION

This study indicates that an enhanced vulnerability to alcoholism may be associated with a profoundly reduced \(G_s\)-protein stimulated adenylyl cyclase activity in platelets, providing a sensitive biochemical trait marker for a predisposition to alcoholism.
It is known that ethanol exposure causes an immediate stimulatory effect on the ubiquitous membrane-bound enzyme adenylyl cyclase by activation of Gs-proteins. Such an acute biochemical effect of ethanol is apparent both in the central nervous system and in the periphery.\textsuperscript{6,18,19} In discrete brain areas such as hippocampus and caudate nucleus as well as in the platelets, the type VII adenylyl cyclase is abundant and showed to be particularly sensitive to activation by ethanol.\textsuperscript{19-21} An opposite effect of ethanol on adenylyl cyclase activity was observed after chronic ethanol exposure.\textsuperscript{6} Studies in cerebral cortex membranes of chronically ethanol-fed mice showed that the reduction in adenylyl cyclase activity is not caused by changes in the catalytic unit of the enzyme.\textsuperscript{22} It appears more likely that the cellular adaptive effect is caused by a reduction in gene expression of the ã-subunit of Gs-protein, as demonstrated in ethanol exposed neuroblastoma cells.\textsuperscript{23} In agreement with this hypothesis, a reduction in the number of Gs-protein was found in a post-mortem study of brains of alcoholics.\textsuperscript{9}

So far, there is no evidence that either the acute stimulatory effect of ethanol on adenylyl cyclase or the adaptive decrease in adenylyl cyclase activity in the brain upon chronic ethanol exposure plays a role in the pathophysiology of alcohol addiction. Nevertheless, similar effects in platelets could provide us with a simple trait marker for a predisposition to alcoholism. Apparently, prior ethanol abuse may lead to a persistent reduction in Gs-protein stimulated adenylyl cyclase activity in non-neuronal cells such as platelets as demonstrated in the central nervous system.\textsuperscript{9} A reduced Gs-protein functioning was reported in platelet membranes of alcoholics\textsuperscript{5,8} even after 90 days of ethanol abstinence.\textsuperscript{7} Although these findings are consistent with the hypothesis that platelet Gs-protein functioning represents a trait marker for enhanced vulnerability to
alcoholism, most studies performed thus far involved experiments on tissues obtained from alcoholics. Therefore, the observed reduction in cAMP formation by platelet membranes of alcoholics could simply be due to a persistent cellular effect of previous alcohol exposure. Therefore, it is crucial to test this hypothesis using platelet membranes of people that do not regularly consume ethanol.

Because COAs are well known to be at high risk for future alcoholism, these children constitute an excellent population to evaluate the validity of the trait marker hypothesis. Considering the value of this experimental group, it is of interest that Devor et al11 provided evidence for a possible Mendelian genetic transmission of fluoride-induced (G_s-protein mediated) adenylyl cyclase activation in platelets. Therefore, if this hypothesis holds true, one would expect that the fluoride-stimulated cAMP production in platelets of COAs is considerably lower than that in platelets of control children of non-alcoholic relatives. Indeed, we found such a lower fluoride induced activation of adenylyl cyclase in platelet membranes of our male and female COAs. In contrast, there was no difference in cAMP production induced by direct activation of adenylyl cyclase with forskolin between platelet membranes of COAs and platelet membranes of control children, indicating altered G_s-protein functioning. Moreover, whereas Saito et al7 primarily observed reduced G_s-protein functioning in platelets of alcoholics with an alcoholic first degree relative, we observed a 50% lower fluoride-stimulated adenylyl cyclase activity in platelets of FHP-multi COAs than COAs with first-degree alcoholism. In view of our present results, we will perform a follow up study to assess the degree of correlation between fluoride-stimulated adenylyl cyclase in platelet membranes assessed in the present study and future alcohol abuse.
In conclusion, our data show that \( G_s \)-protein stimulated adenylyl cyclase activity in platelet membranes of FHP-multi COAs is about 50% lower than that in platelet membranes of control children. Because (1) these young children are well known to be at high risk to develop alcoholism in the near future, (2) our COAs did not consume ethanol more frequently than control children, and (3) these children had a similar reduction of \( G_s \)-protein-stimulated adenylyl cyclase as previously shown in platelets of adult alcoholics\(^7\), our study strongly suggests that reduced fluoride-stimulated adenylyl cyclase activity in platelets represents a gender-independent trait marker for predisposition to alcoholism, rather than a state marker for alcoholism.
REFERENCES


