し，本剤とグルコースを基本とした輸液により意識覚醒の程度をモニタリングする[18]．BCAA輸液は合成二糖類などと比較して完全覚醒までの日数が有意に短く，慢性再発型では速効性の意識覚醒効果を示すことが多いが，末期型での効果は一過性であり，逆に高アンモニア血症や脳症の悪化をきたす可能性もあることから過剰投与は避ける必要がある[19]．

脳症が覚醒して経口摂取が可能になった時点で，肝不全用経腸栄養剤1～2包/日に切り替え，徐々に低蛋白食（0.4～0.6 g/kg標準体重）を上乗せする．BCAA顆粒にはバリン，ロイシン，イソロイシンのみが含有されており，経口摂取可能例における軽度の脳症の改善やアンモニア代謝の是正も期待できる．

c．亜鉛（Zn）の補充

亜鉛は，肝臓の尿素回路におけるオルニチントランスカルバミラーゼ活性や骨格筋のグルタミン合成酵素の活性を維持する目的で投与するが，肝性脳症に対する単独治療としての有効性は明らかにされていないことから[20]，蛋白制限食や合成二糖類などの治療不応例に対する併用療法の一つとして位置付けられている．

従来は，酢酸亜鉛や硫酸亜鉛の内服が一般的であったが，胃潰瘍治療薬として広く使われているポラプレジンク（プロマック®）は調剤の必要がなく，消化器症状も少ないことから，継続的な補充に適している（保険非適応）．

6．入院管理を考慮すべき病態

在宅医療には，「療養」を目的とした診療と「看取りの場」としての診療があるため，個々の病態や患者・家族の意向を踏まえながら入院管理の必要性を考える必要がある．在宅医療が療養の場として位置づけられる場合には，基本的には上述の治療が奏効しない時に入院管理を考慮する．

塩分制限や利尿薬，アルブミン投与により軽減されない，もしくは穿刺排液後も早期に腹水が再貯留するような「難治性腹水」のほか，脳症を繰り返す症例（門脈–大循環短絡の要因が強いタイプ）では専門施設へのコンサルテーションを考慮する．また，特発性細菌性腹膜炎（SBP）の併発や消化管出血による肝性脳症，経口摂取が不十分な状態が続く場合には，速やかに入院治療を考えるべきである．

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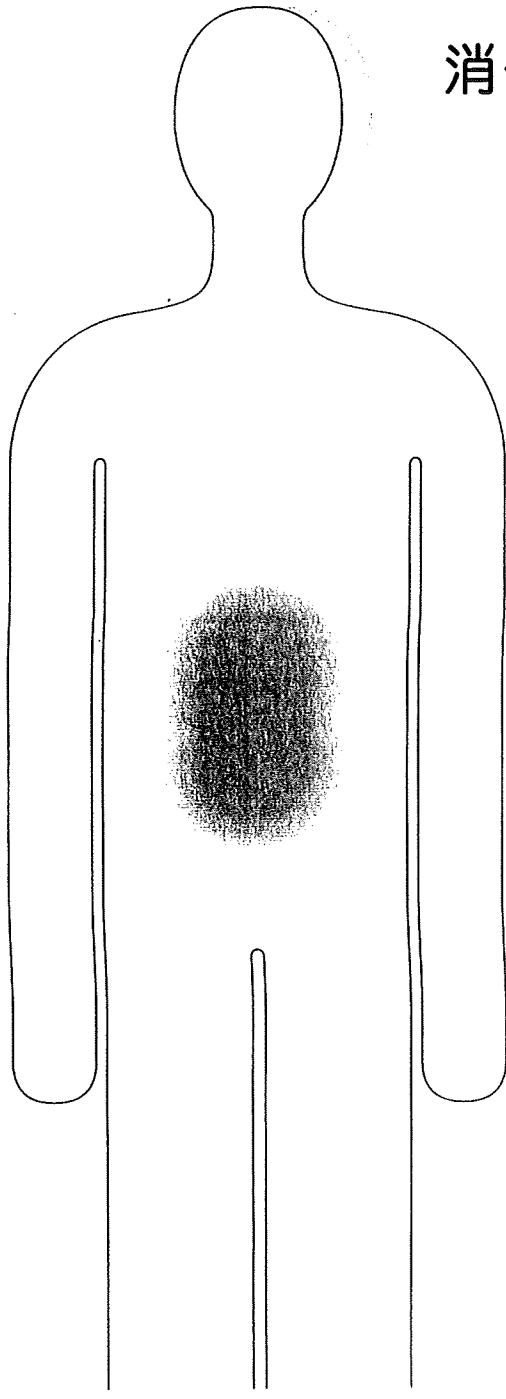
新しい診断と治療のABC 62

アルコール性肝障害

消化器 9



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第4章　アルコール性肝障害の診断と治療

アルコール性肝硬変の診断と治療

要旨

アルコール性肝硬変の診断と治療について解説した．病因の診断には詳細な飲酒歴の問診が必要であるが，C型肝炎の合併例も多いことに注意が必要である．診断は肝組織学的検査とともに理学所見，血液生化学検査，画像検査などにより総合的に行う．治療では禁酒指導が基本であり，そのうえで肝硬変の病期に準じた栄養療法，薬物療法などを行うことが肝要である．

●キーワード

飲酒
アルコール
肝硬変
病期
栄養

はじめに

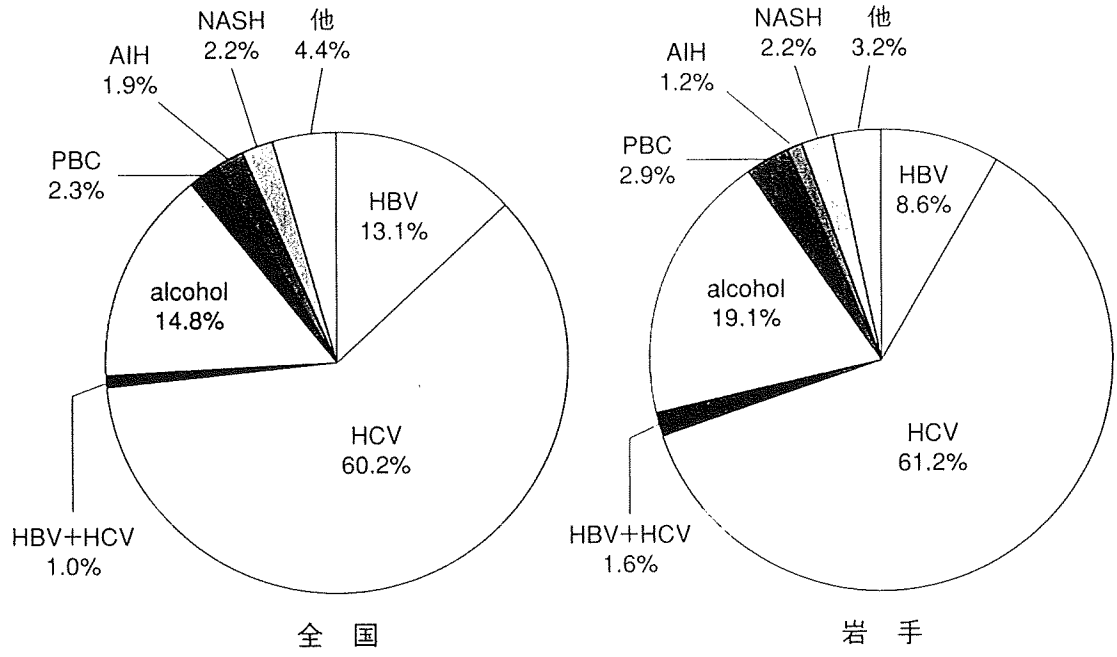
近年，我が国においては生活習慣病への対策が重要な課題となっており，肝疾患では非アルコール性脂肪性肝障害（NAFLD）が注目されてきている．しかしながら，生活習慣病の代表的な危険因子である飲酒によるさまざまな健康問題も未解決であり，アルコール性肝疾患の罹患率も減少していない．

本稿ではアルコール性肝硬変の診断と治療について述べる．

アルコール性肝硬変の頻度

第 44 回日本肝臓学会総会において，過去 10 年間の我が国における「肝硬変の成因別実態」が集計された．合計 33,379 例のうち，アルコール性は 13.6％（男性：19.2％，女性：4.3％）でC型肝炎ウイルス（HCV），B型肝炎ウイルス（HBV）に次いで第3位の頻度であった．その頻度は，過去2回の集計と比較するとほぼ同頻度であり，減少傾向は認めなかった．また，その他の傾向としては，男性に多く，東北，北海道に多くみられた[1]．自験例では，1999 年から 2008 年までに岩手医科大学附属病院肝臓内科に入院し肝硬変と診断した 823 例を対象に検討した結果，頻度においてはアルコールを病因とする症

図1　1999～2008年における肝硬変の成因別頻度



1999年から2008年までの肝硬変の成因を示す．全国の症例数の合計は33,379例（男20,817例，女12,562例）で58施設．岩手の症例数は，823例（男535例，女288例）を対象とした．なお，アルコール＋ウイルス性タイプは各ウイルス群に含めた．

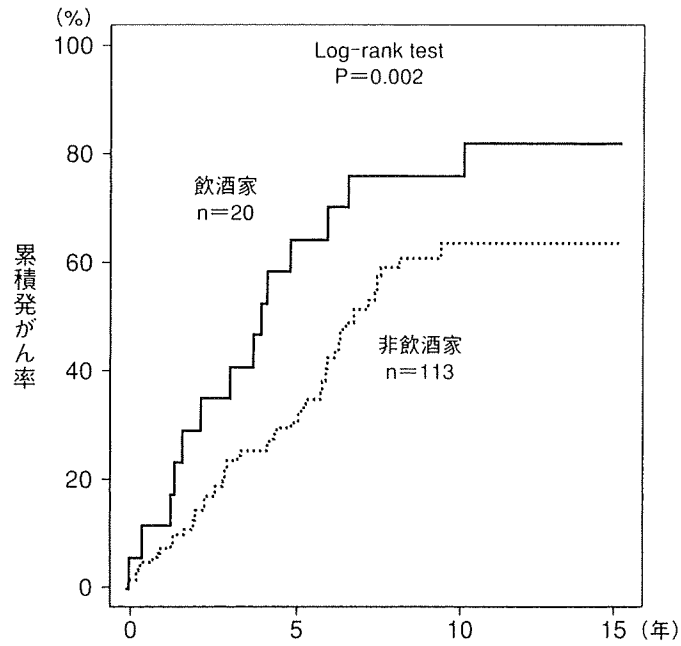
AIH：自己免疫性肝炎，PBC：原発性胆汁性肝硬変，NASH：非アルコール性脂肪性肝炎，HBV：B型肝炎ウイルス，HCV：C型肝炎ウイルス

例が157例（19.1％）でC型肝硬変に次いで第2位であった（図1）．性差においては，男性141例，女性16例で，有意に男性に多かった[2]．全国，岩手共に肝硬変の病因としてのアルコールの占める割合は，増加傾向にあるものと思われる．

アルコール性肝硬変の診断

肝硬変は形態的な診断名であり，確定診断には病理所見（びまん性の肝線維化と結節形成）が必要であるが，形態学的証拠が得られなくとも，画像所見，臨床所見から典型例では肝硬変の正確な診断が可能である．一方，大酒家の肝硬変ではHCV抗体陽性例も多く，肝障害自体がウイルス，アルコールいずれによる影響が大きいかを区別することは容易ではない．したがって，肝硬変は病因的に「アルコール性」と「アルコール＋ウイルス性」の2型に分けて考える必要がある．その理由として，「アルコール＋ウイルス性」は発がん率が高いことが

図2　飲酒家C型肝硬変の累積発がん率



1日平均日本酒換算3合以上を飲酒家とし，一方，3合以下あるいはまったく飲酒をしないものを非飲酒家として検討した．飲酒家と非飲酒家との間ではC型肝硬変からの肝細胞癌累積発がん率に有意差を認めた．

報告されており[3][4]，自験例においても，多量飲酒を伴うC型肝硬変群の累積発がん率は，C型肝硬変群に比べ有意に高い（図2）．

「アルコール性」と「アルコール＋ウイルス性」の診断は飲酒歴，禁酒による肝逸脱酵素の変化，特徴的な臨床所見，血液生化学検査，画像所見や肝生検などにより行う．特に，飲酒歴の聴取は重要であり，量，種類，期間などについて，患者の生活様式，食事摂取量におけるアルコール摂取の占める割合について正しく知るため，詳細な問診が必要である．また，正確な飲酒歴の聴取には患者本人だけでは十分ではなく，家族からの聴取も大切である．機能的には代償性と非代償性に分類されるが，非代償性肝硬変では，特にアルコールとウイルスのいずれが病因の主体になっているかを判断できない例が多く，このような例では大酒家非代償性肝硬変と一括し，ウイルスマーカーの有無を付記する．高田班[5]によるアルコール性肝障害の診断基準案（表1）では3合／日以上を常習飲酒家，5合／日以上を5年以上継続した場合を大酒家とし，これらに伴う肝障害をアルコール性肝障害とした．欧米でも一般的に80g／日以上の飲酒家は肝障害のリスクが高まるとされている[6][7]．ただし，C型肝炎ウイルス感染者はこの限りではなく，また女性は男性に比べ少量の飲酒でも肝障害を起しうることが知られており，上記飲酒量の2／3程度とする．清酒1合に相当するアルコール飲料の濃度と量を示す（表2）．

身体所見ではアルコール臭，圧痛を伴う肝腫大，黄疸，くも状血管腫，手掌紅斑，頬部や鼻尖部，前胸部にかけての毛細血管の拡張，腹水，脾腫などが診断の手がかりとなる．血液生化学検査としては，禁

表1　文部省「高田班」によるアルコール性肝障害の新しい診断基準（抜粋）
（文献[5]より引用改変）

概　念

「アルコール性」とは，長期（通常5年以上）にわたる過剰の飲酒が肝障害の主な原因と考えられる病態で，以下の条件を満たすもの．

A．「アルコール性」

1．常習飲酒家（1日平均3合以上），または大酒家（5合以上，5年以上継続）である．ただし，女性は2/3の程度の飲酒量，ALDH2活性欠損者では3合以下でも，アルコール性肝障害を生じうる．

2．禁酒により血清AST，ALT活性が明らかに改善し，4週以内にほぼ正常化する．

3．肝炎ウイルスマーカーは陰性である．

4．次の検査のうち，少なくとも1つが陽性である．

1）禁酒による肝腫大の著明な縮小，4週でほぼ正常化．

2）禁酒による血清γ-GTP活性の明らかな低下．

5．以下のアルコール性肝障害に特異的なマーカーが陽性なら，より確実．

1）血清トランスフェリンの微小変異陽性

2）CTスキャンによる肝容量の増加

3）アルコール肝細胞膜抗体陽性

4）血清GDH，OCTが異常高値でGDH/OCT＞0.6以上．

B．「アルコール＋ウイルス性」

肝炎ウイルスマーカーが陽性で，上記Aの2を除き，上記Aの条件を満たす．

C．「その他」

上記の条件を満たさない場合は，大酒家であっても「アルコール性」あるいは「アルコール＋ウイルス性」と確診することは困難．ただし，禁酒後の変化が十分に追跡できなくても，アルコール性肝障害に典型的な組織所見が得られた場合には「アルコール性」ないしは「アルコール＋ウイルス性」とする．

略語：巻末の略語集参照

酒により改善するアスパラギン酸アミノトランスフェラーゼ（AST）優位の肝逸脱酵素上昇（4週間後に前値の40％以下か，前値が100 IU/L以下の場合は正常値まで改善），γ-グアノシン5'-三リン酸（γ-GTP）高値（4週間後に前値の40％以下か，正常値の1.5倍以下まで）を認める．その他，中性脂肪，尿酸，免疫グロブリンA（IgA）などの高値を伴う．また，線維化マーカーとしてPⅢPや血清ヒアルロン酸，Ⅳ型コラーゲン7sが用いられているが，アルコール性肝硬変では個々のばらつきが大きく，その評価には注意が必要である．また，近年，肝線維化あるいは肝の硬度を超音波など用いて非侵襲的に計測する方法が開発され，その信頼性の評価が進められており，

表2　清酒1合に相当するアルコール飲料の濃度と量

清酒（14 ～ 16 %）	1 合	180 ml（22 g）
ウィスキー（43 %）	ダブル 1 杯	60 ml（21 g）
ワイン（12 %）	2 グラス	220 ml（28 g）
ビール（4.4 %）	大瓶 1 本	630 ml（22 g）
焼酎（25 %）	コップ 1 杯	100 ml（25 g）

表3　文部省「高田班」によるアルコール性肝障害の分類（抜粋）
（文献[3]より引用改変）

「アルコール性肝硬変」
1．肝組織病変が小結節性，薄間質性である．
2．病因的に「アルコール性」と「アルコール＋ウイルス性」の2型に分けられる．
3．組織学的に証明を欠く場合には，肝硬変（臨床的）とする．
4．機能的には代償性と非代償性に分類する．非代償性肝硬変でアルコールとウイルスのいずれが病因の主体か判断できない例では，大酒家非代償性肝硬変として一括し，ウイルスマーカーの有無を付記する．

今後の診断に期待される[8]．

肝生検は診断と病期の確定，予後を判断するうえで重要である．中心静脈周囲（zone 3）の線維化の所見は，肝硬変への進展を予測するうえで有用である．既述した高田班の診断基準では，肝生検所見により非特異的変化群，アルコール性脂肪肝，アルコール性肝線維症，アルコール性肝炎，大酒家慢性肝炎，アルコール性肝硬変，大酒家肝癌など各病型に分けている（表3）[3]．しかしながら，全例に肝生検を施行することは難しいため，実際には身体所見，血液生化学検査と腹部 CT 検査，腹部超音波検査，肝シンチグラムなど画像所見を組み合わせて診断を確定する場合が多い．

なお，肝硬変の重症度判定は Child-Pugh score（表4）[9] を用いて行い，治療法の選択，予後予測，肝移植適応の判定などに活用する．

アルコール性肝硬変の治療

1．一般療法

アルコール性肝硬変の治療の基本は禁酒であり，その他の治療法は補助的な治療法であることを認識すべきである．しかしながら，多くの例は，アルコール性肝障害を指摘されたときより禁酒指導を受けているにもかかわらず，継続飲酒し肝機能の増悪・軽快を繰り返していたことが推察される．あらためて，禁酒の重要性を患者に説明し，あるいは家族の協力も得て指導し，精神科医に禁酒を目的とした受診を勧めることも重要である．

アルコール性肝硬変は代償期，非代償期にかかわらず，栄養不良を伴っている場合も多く，食事・栄養療法は必須である．特に，肝硬変

表4　Child-Pugh score（文献[9]より引用）

スコア	1	2	3
血清ビリルビン（mg/dl）	2.0 未満	2.0 ～ 3.0	3.0 超
血清アルブミン（g/dl）	3.5 超	2.8 ～ 3.5	2.8 未満
腹水	なし	コントロール可能	コントロール困難
プロトロンビン時間（%）*	80 % 以上	50 ～ 80 %	50 % 以下
脳症	なし	軽度（Ⅰ～Ⅱ）	重症（Ⅲ～Ⅳ）

*：原著では延長秒数で表示されているが，我が国の実態に合わせて活性値で表示．
総合評価はグレードA：5～6点，グレードB：7～9点，グレードC：10～15点として判定．

に伴う肝性脳症の予防や改善，浮腫や腹水に対する，塩分・水分制限，血糖コントロールなどが重要であり，低栄養状態の改善は肝不全用経腸栄養剤，分枝鎖アミノ酸（BCAA）顆粒剤の併用を考慮する．

具体的には，代償期の肝硬変では外来における治療が中心であるが，禁酒と規則正しい食生活（カロリー：30～35 kcal/kg/日，タンパク：1.0～1.5 g/kg/日）を指導する．腹水などを認める非代償期の肝硬変では，水分制限（1 L/日以下），塩分制限（3～6 g/日）とする．肝性脳症，高アンモニア血症の存在する場合は低タンパク食（タンパク：0.5～0.7 g/kg/日＋肝不全用経腸栄養剤）とする．十分な経口摂取が可能な例でかつ低アルブミン血症（3.5 g/dl 以下）を認める例ではBCAA顆粒剤の投与も行う[10]．

当科では，個別栄養指導のほか，肝臓栄養指導教室を行い患者が繰り返し勉強できるような環境を整えている．また，栄養サポートチーム（NST）の介入が可能な施設では積極的に導入することを推奨する．

2．薬物療法

薬物療法は補助的なものであるが，polyene phosphatidylcholine（EPL），肝庇護薬（グリチルリチン製剤，ウルソデオキシコール酸など）の投与を行う．近年ビタミンEがアルコール性肝障害の進展を抑制するという報告[11]があり，臨床的エビデンスの確立が期待される．ビタミン，微量元素（亜鉛）動態をモニターし，補充が必要な例では適時加え，プロトロンビン時間が延長する例にはビタミンKも投与する．腹水を伴う場合は，利尿薬（抗アルドステロン薬，ループ系利尿

薬）の投与が必要となる．難治性腹水の場合，生活の質（QOL）改善目的に腹水濃縮再静注療法，肝内門脈大循環シャント，腹膜静脈短絡術（Denver Shunt）の導入を検討する．

肝移植が一般化している欧米では，アルコール性肝硬変患者に対する肝移植は，ほかの肝疾患に対するものと同等ないしそれ以上の成績を上げている．一方，我が国の脳死肝移植におけるレシピエント選択基準[12]では，アルコール性肝硬変はⅢ群に分類されており対象疾患としての優先度は低い．しかしながら，生体肝移植では，ドナーの確保が可能であれば検討すべき治療法である．移植時期については，前述した Child-Pugh score のほか，Model for End-Stage Liver Disease（MELD）score を参考にすることが推奨されている．MELD score は肝硬変の短期予後を的確に反映することから，肝移植時期の決定に応用されるようになり，全米臓器配分ネットワーク（UNOS）の臓器配分基準に採用されている．また，最近は肝硬変にみられる低ナトリウム血症が予後と深くかかわっていることが示され，これを加味した MELD-Na score も提唱されている．いずれも Mayo clinic のホームページに，自動的に計算できるサイトが提供されている（http://mayoclinic.org/meld/mayomodel15.html）[13]．1年生存を待機例と移植例で比較した成績から，一般にスコア 15 程度以上が移植適応と考えられている．ただし，欧米で一般的に行われている脳死肝移植と日本で行われている生体肝移植では適応の考え方が異なるため，慎重な対応が必要と考えられる．また，6ヵ月間禁酒を継続できた症例に肝移植を行うという考え方を提案している報告もある[14]．

最後に，アルコール性肝硬変においても肝細胞癌の合併をみることから，定期的な画像検査を怠らないことも重要である．

おわりに

アルコール性肝硬変には医学生物学的面だけでなく，社会的背景，精神的背景などの要素も多分に含まれており，飲酒に関する適切な啓蒙活動をしない限り今後も減少は期待されないと考える．現実的には，アルコール飲酒がもたらす正の側面もあることから，いわゆる“適度なアルコールとの付き合い”が理想ではあるが，それを逸脱するケースは後を絶たない．治療においては，内科医を始めとして外科，精神

科，医療ソーシャルワーカーや看護師，保健婦などのいろいろな分野の専門家とのチーム医療，そして患者の家族と協力する必要があると思われるが，家族を持たないケースがより課題である．

及川寛太・黒田英克・柿坂啓介
及川純子・葛西和博・遠藤龍人
滝川康裕・鈴木一幸

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雑　　誌

Neurochemistry International 55 (2009) 119–123

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Alterations in expression of genes coding for proteins of the neurovascular unit in ischemic liver failure

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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form 26 January 2009
Accepted 27 January 2009
Available online 13 February 2009

Keywords:
Hepatic encephalopathy
Neurovascular unit
Acute liver failure
Blood–brain barrier
Hypothermia
Gene expression
Nitric oxide synthase
Brain edema

ABSTRACT

There is evidence to suggest that integrity of the neurovascular unit may be compromised in acute liver failure (ALF). In order to address this issue from a molecular standpoint, expression of an array of genes coding for key cerebrovascular endothelial cell and tight junction proteins were measured by reverse transcription-polymerase chain reaction in cerebral cortex of rats with ischemic liver failure resulting from hepatic devascularization (portacaval anastomosis followed 24 h later by hepatic artery ligation) compared to appropriate sham-operated controls. Expression of P-glycoprotein, endothelin-1, von Willebrand factor, caveolin-1, occludin, and the endothelial nitric oxide synthase isoform (eNOS) were measured in brain extracts from rats with ALF at coma/edema stages of encephalopathy. The effects of mild hypothermia (35 °C) sufficient to prevent cerebral edema in ALF animals on the expression of these genes were also studied. Brain edema and hepatic coma in normothermic ALF rats was accompanied by selective increases in expression of eNOS. Expression of occludin and von Willebrand factor mRNAs were decreased at coma/edema stages of encephalopathy in ALF rats whereas, expression of other cerebrovascular endothelial cell markers endothelin-1, P-glycoprotein, and caveolin-1 were unaffected. Mild hypothermia led to normalization of brain water content and of eNOS mRNA. However, the correlation between increased eNOS expression and encephalopathy/edema grade was poor suggesting the existence of additional mechanisms. These findings underscore the multifactorial nature of brain edema/encephalopathy mechanisms in ALF and question the role of BBB breakdown as a major pathogenetic factor.

1. Introduction

Hepatic encephalopathy (HE) and brain edema leading to intracranial hypertension and brain herniation are serious neurological complications of acute liver failure (ALF). The precise pathophysiologic mechanisms responsible for these complications are not completely understood. One possible mechanism involves alterations of the neurovascular unit and blood–brain barrier (BBB) dysfunction. Histopathological studies reveal discreet alterations of cerebrovascular endothelial cells (Kato et al., 1992; Potvin et al., 1984) as well as altered expression of genes coding for BBB proteins (Shimojima et al., 2008). Moreover, ammonia, a neurotoxin known to accumulate in brain to millimolar concentrations in ALF (Swain et al., 1992) has been shown to cause decreased expression of the tight junction (TJ) protein claudin-12 (Bélanger et al., 2007) and, under certain conditions, ammonia exposure leads to increases in effective pore size of the BBB (McClung et al., 1990).

As part of a series of studies to assess the neurovascular unit in relation to the pathogenesis of HE and brain edema in ALF, expression of an array of genes coding for some key BBB proteins were measured by reverse transcription-polymerase chain reaction in cerebral cortex of rats with ALF resulting from hepatic devascularization compared to sham-operated controls. Expression of the TJ protein occludin and von Willebrand factor (vWF), caveolin-1 (Cav1), P-glycoprotein (P-gp), endothelin-1 (ET-1) and the endothelial isoform of nitric oxide synthase (eNOS) mRNAs were measured in groups of animals maintained at 37 °C compared to a similar group maintained mildly hypothermic (35 °C) sufficient to prevent signs of encephalopathy and to prevent brain edema in these animals (Rose et al., 2000; Stravitz et al., 2008).

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Abbreviations: HE, hepatic encephalopathy; HAL, hepatic artery ligation; ALF, acute liver failure; BBB, blood–brain barrier; ET-1, endothelin-1; eNOS, endothelial nitric oxide synthase; P-gp, P-glycoprotein; vWF, von Willebrand factor; Occ, occludin; Cav1, caveolin-1.

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doi:10.1016/j.neuint.2009.01.023

2. Materials and methods

2.1. Surgical procedures

Adult male Sprague–Dawley rats (200–250 g) purchased from Charles River (Saint-Constant, Quebec, Canada) were routinely tested for common pathogens and were free of infection at the onset of surgery. Rats were anesthetized with isoflurane, and an end-to-side portacaval anastomosis was performed according to the guidelines of Lee and Fisher (1961). Briefly, rats underwent a laparotomy, the inferior vena cava and portal vein were isolated and clamped using an anastomosis clamp (Roboz Instruments Inc., Washington. DC) and an elliptical portion 1.5 times the diameter of the portal vein was removed. The portal vein was ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was <30 min. Sham-operated control rats, matched for weight, were similarly anesthetized and the inferior vena cava was clamped for 20 min. Following surgery all animals were individually housed with free access to food and water under constant conditions of temperature, humidity and light cycles. Twenty-four hours after portacaval anastomosis, rats were reanesthetized and subjected to hepatic artery ligation (HAL). Following HAL, arterial blood glucose levels were monitored and glucose was administered subcutaneously as needed to maintain normoglycemia. Body temperature and reflexes were monitored every 15 min and temperature was maintained at 37 °C ± 0.5 °C by means of heating pads. Hypothermia occurred spontaneously in the absence of external heating and body temperature was maintained at 35 °C ± 0.5 °C using heating pads when necessary. A group of animals was sacrificed 6 h (prior to the appearance of encephalopathy and brain edema) following HAL (ALF-6 h). A second group was sacrificed approximately 13 h following HAL (ALF-37) at the coma stage of encephalopathy (defined as the loss of righting and corneal reflexes) at which time all animals had significant brain edema. Hypothermic animals (ALF-35) were sacrificed in parallel with time-matched comatose normothermic ALF animals and sham-operated controls. Brains were rapidly removed, dissected on ice and were immediately frozen in isopentane. All tissues were stored at −70 °C until use. All the above surgical methods were conducted in accordance with the Guidelines of Canadian Council of Animal care and were approved by Animal Research Committee at Saint-Luc Hospital (C.H.U.M.).

2.2. Brain water measurement

Brains were kept at 4 °C and cut into 2-mm slices. 1-mm punch biopsy specimens were obtained from the gray matter of the cerebral cortex. Water content of each specimen was measured gravimetrically using a density gradient of bromobenzene–kerosene precalibrated with K_2SO_4 as previously described (Marmarou et al., 1978). The cortical samples were placed onto the fluid column and the equilibration point was measured within 2 min. The specific gravity of the tissue was calculated and results expressed as percentage of water content. Eight measurements were made per animal, and values were arithmetically averaged.

2.3. RNA extraction

Total RNA was extracted using TRI Reagent (MRC Inc., OH) according to the manufacturer's protocol. Putative contaminating DNA was eliminated by adding 100 U of RNase-free DNase I per 50 μg of total RNA at 37 °C for 1 h. Purified RNA was then extracted with phenol, precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. RNA samples were kept at −70 °C until use.

2.4. Semi-quantitative reverse transcription-polymerase chain reaction

Expression of eNOS, occludin, caveolin, P-glycoprotein, von Willebrand factor, and endothelin-1 was investigated by standard one-step semi-quantitative RT-PCR. Total RNA (1 μg) was mixed with 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% bovine serum albumin, 200 μM dNTPs, primers at 1 μM each, AMV reverse transcriptase (80 U/ml), *Taq* DNA polymerase (20 U/ml) and 50 μCi/ml [α ^{32}P]dCTP (3000 Ci/mmol), for a total reaction volume of 50 μL. The reactions were initially heated at 50 °C for 20 min followed by PCR at 95 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min. Amplification efficiency conditions were determined after a kinetic study to ensure all experiments were performed in the exponential phase of amplification where PCR products remain proportional to initial template concentration (data not shown). In all the experiments, β-actin was used as an internal standard to monitor loading variations. After amplification, samples were electrophoresed onto 9% polyacrylamide gels, dried, autoradiographed at −70 °C with an intensifying screen. Each band was excised and Cerenkov radiation was quantified using a β-counter.

Oligonucleotide primers were designed using the PRIMER3 program (Rozen and Skaletsky, 2000) at http://primer3.sourceforge.net/based on the following GeneBank accession numbers: V01217 (β-actin), X59949 (eNOS), XM_342759 (von Willebrand factor), M64711 (endothelin-1), BC161826 (Caveolin-1), AB016425 (Occludin), and L15079 (P-glycoprotein). The forward and reverse oligonucleotide primer sequences were as follows: 5′-CATCCCCCAAAGTTCTAC-3′ and 5′-CCAAAGCCTTCATACATC-3′ (β-Actin, 347 bp); 5′-TCAGCGGCTGGTACATGAG-3′ and 5′-ACAGGAAATAGTTGAC-CATCTC-3′ (eNOS, 351 bp); 5′-TGCTTCTTACGCCCATCTCT-3′ and 5′-CACTCA-TACTCTGGGCAGCA-3′ (von Willebrand factor, 444 bp); 5′-AGTGTGTCTACTTCTG-CCAC-3′ and 5′-CAGCACTTCTTGTCTTTTTGG-3′ (Endothelin-1, 178 bp); 5′-ACCGCTT-GCTGTCTACCATC-3′ and 5′-ATCTCT TCCTGCGTGCTGAT-3′ (Caveolin 1, 235 bp); 5′-GCTTTAATCATTGTTTTTGCTGTG-3′ and 5′-CTCTAGGTTATCGTTGCTGCTGTA-3′ (Occludin, 357 bp); 5′-CTTTGTGGTGGGGACACTCT-3′ and 5′-CGTCTGTGGCGAGTCT-TGTA-3′ (P-glycoprotein, 332 bp). The specificity of the oligonucleotide primers was verified using the program BLASTN (National Center for Biotechnology Information, Bethesda, MD).

2.5. Statistical analysis

All data are expressed as the mean ± S.E.M. and statistical analysis was performed using unpaired Student's *t*-test (2 group comparisons) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis (multiple comparisons). A probability of $p < 0.05$ was chosen to establish significance between the groups. Data were analyzed by using Prism 4.0 software (Prism 4.0, San Diego, CA).

3. Results

Following hepatic devascularization, normothermic animals developed symptoms of encephalopathy progressing from lethargy to loss of righting and corneal reflexes (coma stage). Hypothermia significantly delayed the onset of encephalopathy so that at the time normothermic rats were comatose, hypothermic animals had not started to show significant neurological deterioration. Rats sacrificed at coma stages of encephalopathy had significantly higher brain water content ($p < 0.001$) while paired rats kept mildly hypothermic (35 °C) had brain water content equivalent to that of sham-operated control animals (Fig. 1).

RT-PCR analysis revealed a significant 1.5-fold ($p < 0.05$) increase in the steady-state level of eNOS mRNA occurring as soon as 6 h (precoma) following hepatic devascularization and reaching maximal levels at coma stage of encephalopathy (1.6-fold, $p < 0.001$). However induction of eNOS mRNA expression was prevented in hypothermic animals ($p < 0.01$) (Fig. 2).

In contrast to eNOS, expression of the mRNA encoding the tight junction protein occludin was decreased twofold ($p < 0.001$) in normothermic ALF rats at coma stages of encephalopathy but was insensitive to hypothermia (Fig. 3). Similarly, expression of von Willebrand factor, a multimeric adhesive glycoprotein, was decreased 1.5-fold ($p < 0.01$) in normothermic ALF rats at coma stages of encephalopathy and this decrease was also insensitive to hypothermia (Fig. 4). Expression of the structural protein caveolin-1, endothelin-1 and the multidrug resistance P-glycoprotein were not altered in ALF rats irrespective of body temperature (Figs. 5–7).

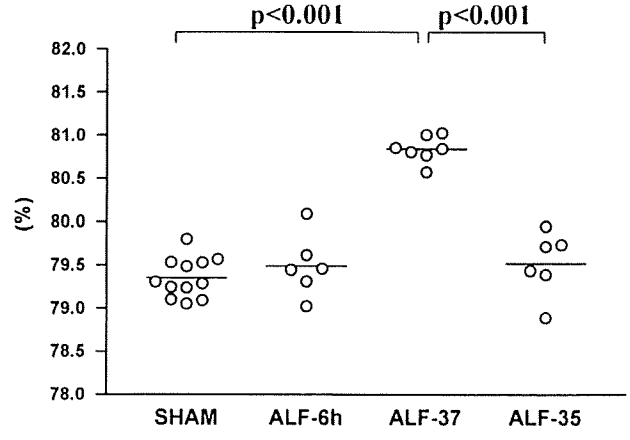

Fig. 1. Normalization of water content of cerebral cortex in ALF rats by mild hypothermia. Brain water content in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic ALF rats (ALF-37) had significantly higher ($p < 0.001$) brain water content compared to sham-operated controls (SHAM), ALF rats 6 h post-HAL (ALF-6 h) or hypothermic ALF rats (ALF-35). Data points represent individual animals and horizontal bars indicate mean values of $n = 6$ animals per treatment group.

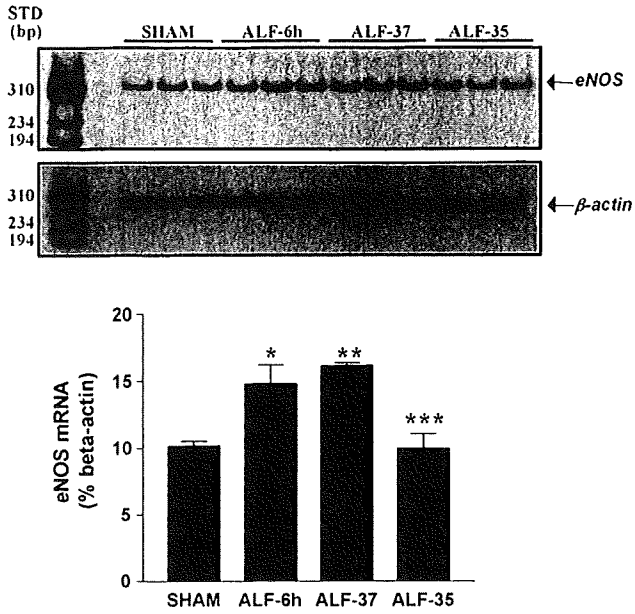

Fig. 2. Increased expression of eNOS in cerebral cortex and its prevention by mild hypothermia in experimental ALF. Endothelial nitric oxide synthase (eNOS) mRNA expression in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic ALF rats (ALF-6 h, ALF-37) had significantly higher steady-state levels of eNOS mRNA compared to sham-operated controls (SHAM) or hypothermic rats (ALF-35). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. sham-operated controls; $^{***}p < 0.01$ vs. ALF-37.

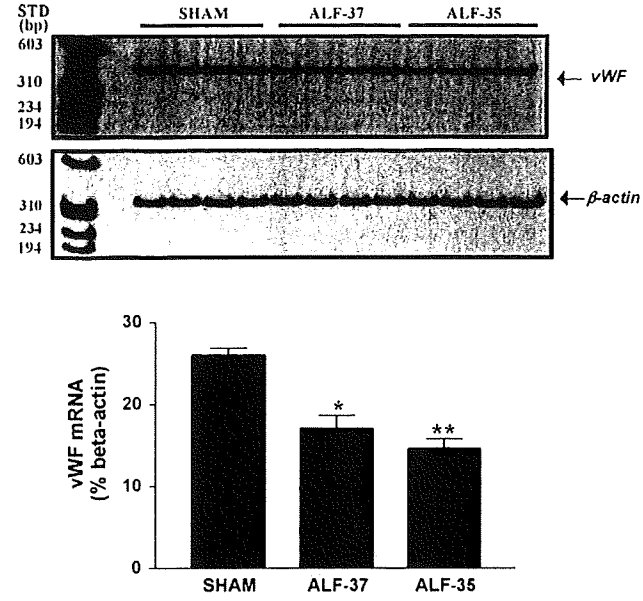

Fig. 4. Loss of expression of van Willebrand factor in cerebral cortex in experimental ALF. Expression of von Willebrand factor (vWF) mRNA in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic (ALF-37) and hypothermic (ALF-35) ALF rats had lower steady-state levels of vWF mRNA compared to sham-operated controls (SHAM). $^{*}p < 0.01$ and $^{**}p < 0.001$ vs. sham-operated controls.

4. Discussion

Results of the present study reveals that ALF due to hepatic devascularization leads to selective alterations in expression of genes coding for key proteins of the neurovascular unit and BBB. Coma stages of encephalopathy were accompanied by increased expression of eNOS and decreased expression of the TJ protein occludin and von Willebrand factor. No significant alterations in expression of endothelin or caveolin-1 were evident in the brains of ALF rats at comparable stages of encephalopathy. The precise mechanisms responsible for increased expression of eNOS in ALF are not completely understood. However, a previous study demonstrated that ammonia could be implicated since increased eNOS immunoreactivity was reported in the brains of portacaval shunted rats administered ammonia infusions (Blei, 2005) as well as in rats with thioacetamide-induced ALF (Hernández et al., 2004) both of which manifest brain edema and severe encephalopathy progressing to coma. Increased eNOS has the potential to cause

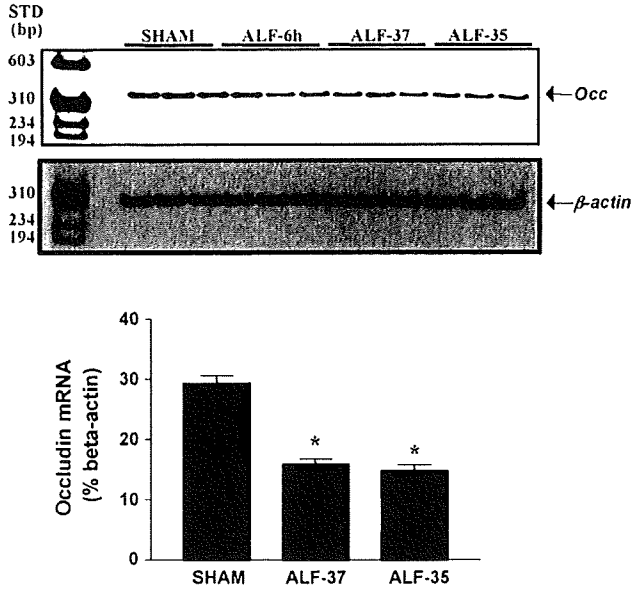

Fig. 3. Loss of expression of occludin in cerebral cortex in experimental ALF. Expression of occludin (Occ) mRNA in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic (ALF-37) and hypothermic (ALF-35) ALF rats had significantly lower steady-state levels of Occ mRNA compared to sham-operated controls. $^{*}p < 0.001$ vs. sham-operated controls.

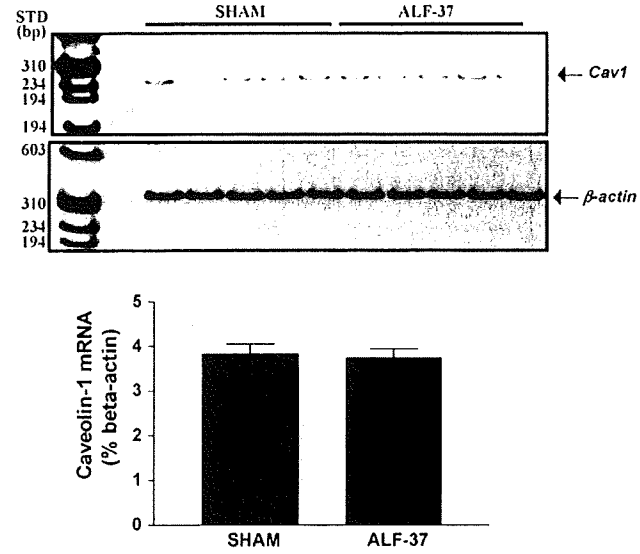

Fig. 5. Expression of caveolin in cerebral cortex in experimental ALF. Expression of Caveolin (Cav) mRNA in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic ALF rats (ALF-37) had similar steady-state levels of Cav mRNA compared to sham-operated controls (SHAM).

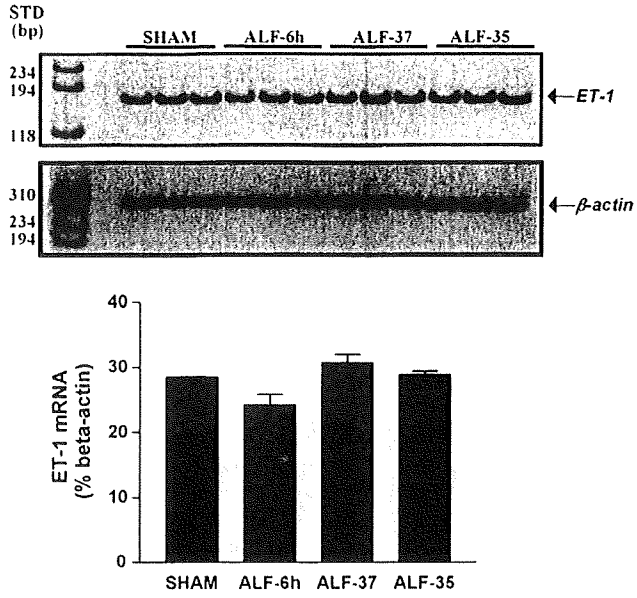

Fig. 6. Expression of endothelin-1 in cerebral cortex in experimental ALF. Expression of endothelin-1 (ET-1) mRNA in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic ALF rats (ALF-6 h and ALF-37) had similar steady-state levels of ET-1 mRNA compared to sham-operated controls (SHAM) or hypothermic rats (ALF-35).

increased production of nitric oxide (NO), vasodilatation and cerebral hyperemia. Increased cerebral blood flow results in increased delivery of ammonia to the brain (Ott and Larsen, 2004), a phenomenon that has been implicated in the pathogenesis of brain edema in ALF (Blei, 2001). In favor of this mechanism, administration of the vasoconstrictor indomethacin has been shown to normalize cerebral perfusion and prevent brain edema both in ALF rats (Chung et al., 2001) and in patients with ALF (Tofteng and Larsen, 2004).

Mild hypothermia completely prevented encephalopathy and brain edema in the present study and concomitantly led to normalization in expression of eNOS suggestive of a causative role. However, these conclusions are tempered by the finding of a poor

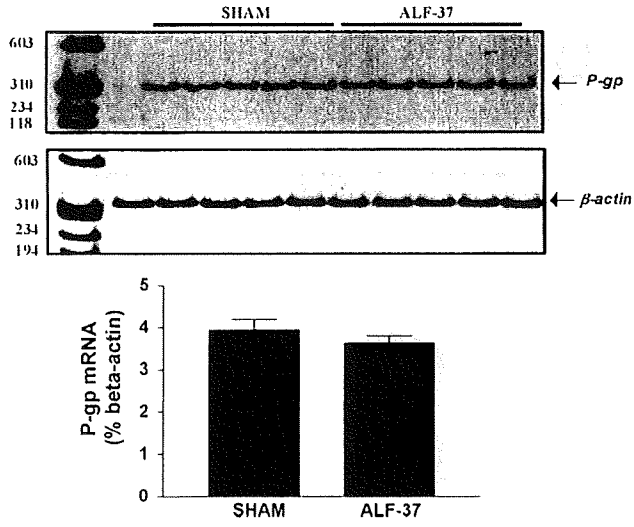

Fig. 7. Expression of P-glycoprotein in cerebral cortex in experimental ALF. Expression of P-glycoprotein P (P-gp) mRNA in rats with ALF due to hepatic devascularization compared to sham-operated controls. Normothermic ALF rats (ALF-37) had similar steady-state levels of P-gp mRNA compared to sham-operated controls (SHAM).

correlation between encephalopathy/edema at the 6 h time point suggesting that, at early stages, factors other than eNOS-derived NO also contribute to the pathogenesis of the early neurological complications of ALF. Such alternative or additional factors include brain glutamine accumulation, increased brain lactate and the presence of proinflammatory cytokines, all of which have the capacity to cause cell swelling and to contribute to the pathogenesis of HE and brain edema in ALF (Albrecht and Norenberg, 2006; Staub et al., 1990; Lazovic et al., 2005).

Loss of expression of the genes coding for the TJ protein occludin and vWF in brain extracts from ALF rats at coma stages of encephalopathy in the present study add to a growing body of evidence that the BBB is dysfunctional in ALF. Although there is no convincing evidence for physical breakdown of the barrier (Larsen et al., 1997), electron microscopic studies previously described discrete changes including swelling of both astroglia and cerebrovascular endothelial cells in the brain of ALF patients (Kato et al., 1992). Morphologic studies in hepatectomized rats reveal increases of vesicular density of the capillary endothelium (Potvin et al., 1984). Previous studies in mice exposed to the hepatotoxin azoxymethane demonstrated decreased expression of the TJ scaffolding protein zona occludens-2 and increased activity of matrix metalloproteinase-9. However, in contrast to the present study, azoxymethane-treated mice manifest clear signs of physical breakdown of the BBB including extravasation of sodium fluorescein and Evans Blue (Nguyen et al., 2006; Shimojima et al., 2008). Moreover, although results of the present study show loss of expression of occludin and vWF in the brain of hepatic devascularized rats, hypothermia sufficient to prevent edema and encephalopathy in these animals did not lead to attenuation of decreased TJ expression suggesting that these changes are not implicated in the pathogenesis of encephalopathy or brain edema in these animals. These findings underscore the notion that in this model of ALF, there is no convincing evidence that breakdown of the BBB and vasogenic edema contribute in a major way to the pathogenesis of HE and brain edema. Similar conclusions were previously reached showing electron microscopic studies in ALF patients (Kato et al., 1992).

In summary, results of the present study demonstrate selective alterations in expression of genes coding for eNOS, as well as the TJ protein occludin and vWF in the brains of rats at coma/edema stage of encephalopathy with ALF due to hepatic devascularization. Further studies will be required in order to determine the corresponding changes in proteins. Mild hypothermia sufficient to prevent encephalopathy and brain edema in these animals led to normalization of expression of eNOS but had no effect on expression of TJ proteins suggesting that cerebrovascular endothelial cell dysfunction but not BBB breakdown was implicated. At early stages of ALF, there was no significant correlation between encephalopathy/edema and increased eNOS expression suggesting the presence of additional (or alternative) mechanisms. Such mechanisms could include brain accumulation of glutamine or lactate as well as the presence of proinflammatory cytokines. These findings underscore the likely multifactorial nature of the mechanisms implicated in the pathogenesis of the neurological complications of ALF and suggest that BBB disruption is not a major feature of ALF due to liver ischemia.

Acknowledgement

This study was supported by a grant from the Canadian Institutes of Health Research.

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Metab Brain Dis (2008) 23:265–274
DOI 10.1007/s11011-008-9092-7

ORIGINAL PAPER

Selective alterations of brain dopamine D_2 receptor binding in cirrhotic patients: results of a ^{11}C-*N*-methylspiperone PET study

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Received: 3 January 2008 / Accepted: 29 May 2008 / Published online: 7 August 2008

Abstract Alterations of the brain dopamine system have been implicated in the neurological complications of chronic liver failure. The present study was aimed at the measurement of dopamine D_2 binding sites in cirrhotic patients by positron emission tomography (PET) using ^{11}C-*N*-methylspiperone as ligand. The regions of interest (ROI) were designated on a three-dimensional stereotaxic ROI template (3DSRT). The pixel values of twelve ROIs corrected by the pixel value of the cerebellum after 80 min static scanning were used to quantitate changes in binding. D_2 binding sites were significantly decreased in the hippocampus and thalamus of cirrhotic patients and were positively correlated with serum bilirubin levels and Child–Pugh scores and were negatively correlated with prothrombin times (thalamus). Loss of D_2 sites was greater in thalamus and hippocampus of alcoholic cirrhotics compared to non-alcoholics. Statistically significant correlations were also observed between D_2 binding sites in hippocampus, thalamus and lenticular nuclei and history of overt encephalopathy. These findings suggest that D_2 receptor binding in some regions of brain in cirrhotic patients is influenced by factors such as the

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severity of liver damage and history of alcohol dependency or overt encephalopathy. Alterations of D_2 receptor sites indicative of dopaminergic synaptic dysfunction could play an important role in the pathogenesis of the cognitive and motor disturbances associated with chronic liver failure.

Keywords Dopamine D_2 receptor · Positron emission tomography · Liver cirrhosis · Hepatic encephalopathy · ^{11}C-*N*-methylspiperone · Alcohol dependency

Introduction

Neuropsychiatric complications of chronic liver disease include sleep patterns and levels of consciousness as well as motor symptoms similar to those observed in Parkinson's disease. These extrapyramidal symptoms are generally believed to be attributable to disorders of dopaminergic neurotransmission in the basal ganglia (Weissenborn and Kolbe 1998; Weissenborn et al. 2000). Previous studies have revealed increased levels of dopamine metabolites (Bergeron et al. 1989) in the brains of cirrhotic patients dying in hepatic coma and reduced dopamine D_2 receptor sites in this same material (Mousseau et al. 1993). However, studies to assess the brain dopamine system in cirrhotic patients *in vivo* have so far been limited to a single case report using SPECT (Weissenborn et al. 2000).

In the present study, we examined dopamine D_2 receptor binding in cirrhotic patients using Positron Emission Tomography (PET) and the dopamine D_2 receptor ligand ^{11}C-*N*-methylspiperone (MSP). Receptor binding in these patients was assessed as a function of the severity of liver disease, patient age, prior episodes of hepatic encephalopathy (HE) and clinical laboratory parameters.

Subjects and methods

Subjects

Twenty eight patients with biopsy-confirmed cirrhosis who presented at Iwate Medical University hospital, between April 2002 and April 2005, were included in the study. Three healthy individuals served as controls, the limited number of healthy controls in the study results from the inherent high cost of PET investigations. Consequently, a stratification paradigm comparing cirrhotic patients with or without exposure to alcohol, prior episodes of HE or ascites was used in the present studies. Informed consent was obtained from all subjects (patients and controls). No patients manifested overt encephalopathy at the time of the study. Patients with overt psychiatric or neurological disorders or receiving treatment with neuroleptic drugs or with any history of exposure to psychoactive/neuroactive medication known to affect the dopaminergic system (antidepressants, antipsychotics, amphetamines, etc.) were excluded. Alcohol dependence was defined as alcohol intake of 75 g per day for five years or more.

Patient profiles are shown in Table 1. The age did not differ significantly between the healthy control subjects (mean±SD: 51.7±16.3; range 34 to 66) and the cirrhotic patients (mean±SD: 58.0±10.2; range: 41 to 75).

Table 1 Characteristics of the 28 cirrhotic patients

Characteristic	*n* (%)[a] [Range]
Age (years)	58.4±10.2[36–66]
Male	18 (64)
Etiology	
Alcohol	9 (32)
Hepatitis C (HCV)	12 (43)
Hepatitis B (HBV)	1 (4)
Alcohol and HCV	4 (14)
Primary biliary cirrhosis	1 (4)
Unknown	1 (4)
Laboratory parameters	
Serum albumin (g/dl)	3.03±0.52
Total bilirubin (mg/dl)	0.86±0.55
Prothrombin time (%)	59.4±20.5
Platelet count ($\times 10^4$ μl)	9.1±5.2
Blood ammonia (μg/dl)	76.8±36.7
Tyrosine (nmol/ml)	136.4±45.6
Phenylalanine (nmol/ml)	91.5±24.5
Branched chain amino acids (nmol/ml)	352.1±166.5
Severity of liver disease (Child–Pugh score)	
A	9 (32)
B	14 (50)
C	5 (18)
History of overt hepatic encephalopathy	6 (21)
History of ascites	11 (39)

[a] Values are means±SD

The study protocol was approved by the Human Ethics Review Committee of Iwate Medical University, Morioka, Japan.

Methods

Blood samples were collected from all patients in the morning after an overnight fast. Biochemical parameters including serum total bilirubin (T.Bil), serum albumin (Alb), platelet count (Plt), blood ammonia (NH_3), prothrombin time (PT%) as well as branched chain amino acids, BCAA (valine, leucine and isoleucine) and aromatic amino acids, AAA (phenylalanine and tyrosine) were measured. The severity of the hepatic function impairment was evaluated according to the Child–Pugh classification (Hanje and Patel 2007).

Dopamine D_2 receptor binding was measured in each region of brain between cirrhotic patients and healthy subjects. Patients were subdivided into groups according to the presence/absence of a history of encephalopathy, alcohol dependence or ascites.

Measurement of biochemical parameters

Peripheral blood counts were measured by routine auto-analyzer (Siemens, ADVIA 120 Hematology Analyzer). Biochemical parameters (T.Bil, Alb, NH_3) were measured by routine auto-analyzer (Biomajesty JCA-BM 2250, JEOL, Ltd.) using commercial kits (Total Bilirubin E-HA; Wako Pure Chemical Industries, Ltd., Aqua-