（東女医倫理委様式1）

倫 理 委 員 会 審 査 申 請 書

平成　22年　1月　29日提出

学　長　殿

申請者名　萩原 誠久　㊞

所　属　名　循環器内科学

職　　名　主任教授

※受付番号＿＿＿＿＿＿

1. 課題名

アミオダロンによる肺障害の予測法の開発

2. 実施（研究）責任者名	所属	職名
萩原 誠久	循環器内科学	主任教授

3. 実施（研究）分担者名	所属	職名
志賀　剛	循環器内科学	准教授
安藤　仁	自治医科大学臨床薬理学	准教授
太田昌一郎	自治医科大学臨床薬理学	講師
牛島健太郎	自治医科大学臨床薬理学	助教
藤村昭夫	自治医科大学臨床薬理学	教授

4. 実施（研究）事項等の概要

アミオダロンは、難治性、致死性不整脈に有効な第Ⅲ群抗不整脈薬であるが、有害反応として肺障害の出現が臨床的に問題となる。しかし、現在のところ、その機序は不明であり、肺障害を十分に予測する方法もない。そこで本研究は、アミオダロンを投与する予定の患者またはアミオダロンによる肺障害の既往のある患者より末梢血を採取し、網羅的遺伝子発現解析を行うことにより肺障害を予測する遺伝子発現マーカーを見出すことを目的とする。

アミオダロンを初めて投与する予定の患者より、本研究への協力の同意を文書にて取得した後に、投与前と投与後早期に末梢静脈血をそれぞれ10 ml採取し、肺障害出現の有無を１年間以上観察する。また、アミオダロンによる肺障害の既往がある（現在は治癒している）患者からも文書にて同意取得後に１回のみ末梢血10 mlを採取する。これらの末梢血液中細胞の遺伝子発現を網羅的に解析し、肺障害出現の有無で発現パターンを比較することにより、肺障害を投与前または投与後早期に予測する遺伝子発現マーカーを探索する。さらに、同定した遺伝子マーカーや遺伝子パスウェイよりアミオダロンによる肺障害の機序を推察し、その解明につなげる。肺障害を予測する遺伝子マーカーが見つかり、そのマーカー遺伝子の発現量（もしくは発現変化）が血清を用いた検査（たとえばマーカー蛋白の定量など）により予測できる可能性が生じた場合には、保存しておいた血清を用いてその検証を行う。

5. 実施（研究）事項等の対象及び実施場所

対象は東京女子医科大学病院循環器内科で診療中の成人患者のうち、アミオダロンの投与を初めて開始することが決まった者、またはアミオダロンによる肺障害の既往がある者、計100名とする。入院・外来の別および性別は不問とするが、本人から本研究への参加の同意が得られる者のみを対象とし、全身状態が悪く同意取得に支障がある場合、高度の貧血（ヘモグロビン濃度＜8 g/dL）がある場合、医師により対象者として不適当と判断された場合は除外する。

検体の採取は大学病院外来または病棟で行い、二重連結可能匿名化した検体および臨床情報を自治医科大学医学部薬理学講座臨床薬理学部門に送付し、遺伝子発現解析は同部門で行う。

注. 実施計画書、インフォームドコンセントの説明書 ・同意書及び審査に必要と思われる参考文献 ・資料等を各１８部添付すること。

注. ※印欄は、記入しないこと。

6. 本課題実施の医学的・医療的意義

アミオダロンは、他剤が無効な致死的不整脈に使用される臨床的に重要な薬物である。しかしながら、肺障害を惹起することがあり、そのために投与を中止せざるを得なくなるばかりか、肺障害が致死的になることもある。したがって、本研究の成果により、アミオダロンの投与前あるいは投与後早期に肺障害出現の予測が可能になれば、アミオダロンの安全性が飛躍的に向上することになり、極めて大きな医療的意義をもたらす。

7. 倫理的配慮について

①実施(研究)等により個人が受ける不利益並びに危険

・本研究に参加しなくてもなんら不利益は受けない。またいつでも自由にやめることができる。

・本研究は日常の診療・治療下で行う。研究対象薬のアミオダロンは、対象患者に治療のために投与されている、あるいは投与予定の医薬品である。

・本研究により提供者に実施される行為は採血のみであり、その量は比較的少なく、高度の貧血を有する患者は除外するため、提供者の危険は十分に小さい。また、本研究による提供者の費用負担はなく、採血は入院中または外来受診時のみに行う(本研究のためだけに来院することはない)ため、特に不利益はないと予測される。

②実施(研究)等の対象となる者に理解を求め同意を得る方法

研究計画の概要について、説明文書により患者に説明する。研究への参加に同意を得た場合には、説明を行った医師名を記載し、同意書に同意年月日、住所の記載と、氏名の自署をしていただく。

③その他(対象となる個人の人権擁護など)

二重連結匿名化 (具体的な方法を記載してください)

自治医科大学へ送付する試料等は、匿名化された状態のもののみとし、個人情報は本学からは一切出さないようにする。

8. その他

※検査の結果	1) 承認 2) 条件付承認 3) 変更の勧告 4) 不承認 5) 対象外		
※倫理委員長印		※通知年月日 平成 年 月 日	※通知番号

臨床試験実施計画書

「アミオダロンによる肺障害の予測法の開発」

第1版　平成21年3月25日作成
第2版　平成22年1月28日作成

1．背景と目的：

アミオダロンは、難治性、致死性不整脈および心房細動に有効な第Ⅲ群抗不整脈薬であるが、有害反応として重篤な肺障害が知られている。[1] アミオダロンによる肺障害は約10%が致死性になるといわれ、その予知は治療を行ううえで重要な課題であるしかし、現在のところ、その機序は不明であり、肺障害を十分に予測する方法もない。さらに日本人では低用量療法であってもアミオダロンによる肺障害が、2.1%/年の頻度に出現している。[2] そこで本研究は、アミオダロンを投与する予定の患者またはアミオダロンによる肺障害の既往のある患者より末梢血を採取し、網羅的遺伝子発現解析を行うことにより肺障害を予測する遺伝子発現マーカーを探索する。

2．研究意義：

アミオダロンによる肺障害出現を予測できれば、危険性が高い患者への投与をあらかじめ避けることが可能になる。さらに、アミオダロンの毒性メカニズムが明らかになれば、肺障害の予防法や肺障害のない薬物の開発につながり、不整脈治療の安全性が飛躍的に向上する。

3．研究方法：

1）対象

東京女子医科大学病院循環器内科で診療中の①または②の患者のうち、以下の選択基準をすべて満たし、かつ、除外基準のいずれにも該当しない者

① アミオダロンの投与が初めて開始される。

② アミオダロンによる肺障害の既往がある（ただし、担当医により治癒したと判断されている）。

【選択基準】

1．年齢：20 歳以上

2．性別：不問

3．入院、外来：不問

4．本人から本研究への参加の同意が文書で得られる。

【除外基準】

1．全身状態が悪く、同意取得に支障がある。

2．高度の貧血（ヘモグロビン濃度＜8 g/dL）が確認されている。

3．その他、医師により試料等提供者として不適当と判断された。

2）方法

アミオダロンを投与する予定の患者より、本研究への協力の同意を文書にて取得した後に、投与前（1ヶ月以内）と投与後早期（投与開始1ヵ月後±2週間）に末梢静脈血を採取

し、肺障害出現の有無を１年間以上観察する。また、アミオダロンによる肺障害の既往がある（現在は治癒している）患者からも文書にて同意取得後に１回のみ末梢血を採取する。提供者の予定人数は合わせて 100 名とする。これらの末梢血液中細胞の遺伝子発現を網羅的に解析し、肺障害出現の有無で発現パターンを比較することにより、肺障害を投与前または投与後早期に予測する遺伝子発現マーカーを探索する。さらに、同定した遺伝子マーカーや遺伝子パスウェイよりアミオダロンによる肺障害の機序を推察し、その解明につなげる。なお、採血は入院中または外来受診時に行い、通常の診療において採血がある場合にはその採血時に追加で研究用検体を採取することとし、本研究のためだけの採血はできるだけ行わないようにする。採取する検体は１回につき、血液 10ml とし、そのうち 5ml を遺伝子発現解析に用い、残りの 5ml からは血清を採取する。

検体は、東京女子医科大学において二重連結可能匿名化した上で保存し、数回に分け匿名化の状態で自治医科大学に送付する。自治医科大学では、遺伝子発現用検体より RNA を抽出し、網羅的遺伝子発現解析は Affymetrix Gene Chip システムを用いて行う。選択した遺伝子群については、real-time PCR 法により mRNA 発現量を確認する。その結果、肺障害を予測する遺伝子マーカーが見つかり、そのマーカー遺伝子の発現量（もしくは発現変化）が血清を用いた検査（たとえばマーカー蛋白の定量など）により予測できる可能性が生じた場合には、保存しておいた血清を用いてその検証を行う。また、遺伝子マーカーが血中薬物濃度に影響している可能性がある場合には、保存血清を用いて薬物濃度測定を行う。

４．研究期間：

許可を得てから平成 24 年 3 月 31 日まで

５．試料等の種類、量、予定人数

種類	静脈血	量	10 ml/回，２回または１回 計 20 ml または 10 ml	予定人数	100 名

予定人数の内訳：新規服薬例　80 名、既服薬者　20 名

６．予測される結果：

アミオダロンによる肺障害の予測が可能になり、アミオダロンの安全性が向上する。

７．予測される危険・不利益：

本研究により提供者に実施される行為は採血のみであり、その量は比較的少なく、高度の貧血を有する患者は除外するため、提供者の危険は十分に小さい。また、本研究による提供者の費用負担はなく、採血は入院中または外来受診時のみに行う（本研究のためだけに来院することはない）ため、特に不利益はないと予測される。

８．個人情報の保護の方法：

提供者の個人情報は、東京女子医科大学にて連結可能匿名化する。対応表は自治医科大学に提供しない。自治医科大学では、匿名化された試料等のみを扱い、個人情報は入手しない。

個人情報管理者：東京女子医科大学　循環器内科学　講師　村崎かがり

９．遺伝情報の開示に関する考え方：

本研究では遺伝子自体は解析しないため、該当しない。また、遺伝子発現解析の結果についても、各個人の結果を試料等提供者に開示することはしない。

１０．研究実施前提供試料等の使用：

なし

１１．試料の提供について：

東京女子医科大学からは匿名化された試料の提供のみ行う。個人情報の提供は一切行わない。自治医科大学以外の機関には提供・委託しない。

１２．試料等の保存：

（1）研究遂行中の試料等の保管

保管責任者の所属・職名・氏名	自治医科大学臨床薬理学　准教授　安藤　仁
保管場所及び方法	自治医科大学臨床薬理学の研究室の冷凍庫に匿名化した状態で保管する。

（2）研究期間の終了後の試料等の保存

採取した血清、血液および抽出・合成された核酸は臨床薬理学において匿名化のまま保存し、本研究期間終了後は直ちに廃棄する。ただし，本研究の解析には長期間を要するため、研究期間終了後に新たな肺障害予測マーカーや肺障害発症機序が推察された場合に備えて、提供者より同意が得られた場合に限り、血清と核酸の保存を継続する。なお、本研究以外の目的で試料を利用する必要が生じた場合には、新たに東京女子医科大学倫理委員会および自治医科大学遺伝子解析研究倫理審査委員会の審査をうける。

（3）ヒト細胞・遺伝子・組織バンクへの試料等の提供

試料等は提供しない。

（4）試料等の廃棄方法及びその際の匿名化の方法

試料等は匿名化された状態でオートクレーブ処理し破棄する。

１３．研究組織、共同研究者の職名、氏名、役割：

東京女子医科大学 循環器内科学　准教授　志賀　剛　　研究（試料等採取）の実施・統括

東京女子医科大学 循環器内科学　主任教授　萩原誠久　　研究（試料等採取）責任者

自治医科大学薬理学講座臨床薬理学部門　准教授　安藤　仁　　遺伝子発現解析

自治医科大学薬理学講座臨床薬理学部門　講師　　太田昌一郎　　遺伝子発現解析

自治医科大学薬理学講座臨床薬理学部門　助教　　牛島健太郎　　遺伝子発現解析

自治医科大学薬理学講座臨床薬理学部門　教授　　藤村昭夫　　研究（遺伝子発現解析等）責任者

１４．参考文献

1. Magro SA, Lawrence EC, Wheeler SH, Krafchek J, Lin HT, Wyndham CR. Amiodarone pulmonary toxicity: prospective evaluation of serial pulmonary function tests. J Am Coll Cardiol. 12:781-8, 1988

2. Yamada Y, Shiga T, Matsuda N, Hagiwara N, Kasanuki H. Incidence and predictors of pulmonary toxicity in Japanese patients receiving low-dose amiodarone. Circ J 71: 1610-1616, 2007

「アミオダロンによる肺障害の予測法の開発」への協力のお願い

はじめに

アミオダロン（商品名 アンカロン）は、重症の不整脈治療に有効な抗不整脈薬であり、他の薬が効かない場合などには必要となる薬です。しかしながら、内服した患者様の数%に、間質性肺炎などの肺障害が副作用として出現することが知られています。現在のところ、この肺障害がどうして起こるのかはわかっておらず、予防法はありません。もしも、アミオダロン治療を始める前、あるいは治療開始後の早い時期に肺障害の危険性が予測できれば、未然にアミオダロンの減量ないし中止をすることが可能になります。また、アミオダロンによる肺障害がどうして起こるのかがわかれば、肺障害を予防する薬や肺障害を起こさない新しい抗不整脈薬を開発することができるかもしれません。

1. 研究の目的

この研究では、アミオダロンの内服を開始される患者様、または、アミオダロンによる肺障害を起こしたことのある患者様を対象に、末梢静脈血 1回 10 ml（2回または1回）を提供いただき、アミオダロンにより肺障害の起こる方と起こらない方の体の状態がどのように異なっているのかを網羅的遺伝子発現解析という方法を用いて調べ、アミオダロンによる肺障害の予測法や予防法を開発することを目的とします。

2. この研究への参加予定期間と参加予定者数

この研究は倫理委員会承認後から平成24年3月まで行なわれ、アミオダロンの処方を受けた患者様（これから初めて内服を開始される方と、以前にアミオダロンで肺障害を起こしたことがあり現在はアミオダロンを中止し肺障害が治っている方）計 100 名の方にご協力いただく予定です。

なお、採血させていただく時期と回数は次のとおりです。

《初めてアミオダロンの内服を開始される方の場合》

①と②の2回

①アミオダロンの内服を始める前1ヵ月以内

②アミオダロンの内服を開始して約1ヵ月後

《アミオダロンによる肺障害を起こしたことがある方の場合》

この研究に同意いただいた後に1回のみ

3. この研究の組織

この研究は、自治医科大学医学部薬理学講座臨床薬理学部門との共同研究で行います。この研究の意義や倫理的妥当性については、東京女子医科大学および自治医科大学において、それぞれの倫理委員会で内容が審査され、その倫理的観点から妥当性が認められまし

た。この研究について何か不明点や相談したいことがありましたら患者様の治療を担当する医師に遠慮なくお尋ねください。

4. 安全性について

患者様にご協力いただくことは通常の方法で行われる腕からの静脈血採血のみであります。その採血量は 10ml であり、高度の貧血を有する患者様には参加をお願いしていません。よって、この研究のために新たに加わる危険性はほとんどありません。

5. 患者様の利益と将来の患者様への利益

この研究にご協力いただく患者様ご自身には直接的な利益はないと考えています。しかし、今後、同じようにアミオダロンの内服が必要になる患者様にとっては、安全な治療を受けられるようになることが期待されます。

6. この研究に関わる費用について

この研究に対して、患者様が費用を負担することは一切ありません。

7. 研究への協力に同意しない場合でも不利益を受けないこと

この研究に協力するかどうかは、患者様の自由意志で決められます。この研究への協力に同意いただけなくても、当院での治療法が異なることはありません。また、このために患者様が診療に不利益を受けることは一切ありません。

8. 研究への協力に同意した場合でも随時これを撤回できること

患者様がこの研究への協力に同意した後であっても、自由に随時これを撤回できます。同意を撤回される場合は担当医に申し出てください。その場合でも患者様が診療に不利益を受けることは一切ありません。

9. プライバシーの保護について

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同　意　書

東京女子医科大学
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私は「**アミオダロンによる肺障害の予測法の開発**」について、研究分担者より文書による説明を受け、研究の意義について理解いたしましたので、この研究に参加することに同意いたします。

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説明日：　　年　　月　　日

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研究成果の刊行に関する一覧表

書籍
なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kumazaki, M., Ando, H., Sasaki, A., Koshimizu, T., Ushijima, K., Hosohata, K., Oshima, Y., and Fujimura, A.	Protective effect of α-lipoic acid against arsenic trioxide-induced acute cardiac toxicity in rats.	**J Pharmacol Sci**	115(2)	244-8	2011
Koshimizu, T., Tsuchiya, H., Tsuda, H., Fujiwara, Y., Shibata, K., Hirasawa, A., Tsujimoto, G., and Fujimura, A.	Inhibition of heat shock protein 90 attenuates adenylate cyclase sensitization after chronic morphine treatment.	**Biochem Biophys Res Commun**	392(4)	603-7	2010
Koshimizu, T., Fujiwara, Y., Sakai, N., Shibata, K., and Tsuchiya, H.	Oxytocin stimulates expression of a noncoding RNA tumor marker in a human neuroblastoma cell line.	**Life Sci**	86(11-12)	455-60	2010

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Short Communication

Protective Effect of α-Lipoic Acid Against Arsenic Trioxide–Induced Acute Cardiac Toxicity in Rats

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Abstract. The clinical use of arsenic trioxide (ATO) is often limited because of its adverse effects. We examined whether α-lipoic acid (LA) protects against the ATO-induced cardiac toxicity. In the chronic study, two of four rats suddenly died by the repeated dosing of ATO, whereas no deaths were observed in combination with LA. In the acute study, continuous ECG recording revealed that intravenous injection of ATO caused transient ST-T change, whereas pretreatment with LA abolished the ATO-induced ECG abnormality in all animals. These results suggest that LA protects against the ATO-induced acute cardiac toxicity and subsequent sudden death in rats.

Keywords: α-lipoic acid, arsenic trioxide, cardiac toxicity

Arsenic trioxide (ATO) is used for the treatment of relapsed or refractory acute promyelocytic leukemia (1). However, the clinical use of ATO is often limited by its adverse effects. For instance, ATO is reported to cause renal injury (2, 3) and cardiac toxicity, including T-wave changes, QT prolongation, torsades de pointes, and sudden cardiac death in humans (4 – 6). We previously performed gene expression analysis using DNA microarrays in human primary renal cortical cells and found that the expression of heme oxygenase 1, which is induced by oxidative stress, was strongly related to the ATO-induced cytotoxicity (7). Moreover, we observed in vitro that α-lipoic acid (LA), a naturally occurring dithiol compound with an antioxidant property (8), ameliorated the ATO-induced cytotoxicity by reducing superoxide anion production, while it did not alter the effect of ATO on promyelocytic leukemia cells or myeloma cells (7). Thus, these observations provide the possibility that LA protects against the ATO-induced adverse effects without loss of therapeutic efficacy in vivo. To determine the characteristics of the ATO-related adverse effects and potential preventive effect of LA, we administered ATO with and without LA repeatedly to small number of Wistar rats. Because the chronic study suggested that ATO caused sudden cardiac death, and this adverse effect was prevented by LA, we further evaluated the effects of ATO and LA on ECG findings in the acute study.

Arsenic trioxide (ATO) was obtained from Nippon Shinyaku Co., Ltd. (Trisenox® injection, Kyoto). (±)-α-Lipoic acid (LA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). For intravenous injection, LA was mixed with distilled water in a dark bottle, and 0.1 N NaOH was added until the solid was dissolved. The pH of the solution was then brought to 7.4 with 1.0 N HCl.

Male Wistar rats were obtained from Japan SLC (Shizuoka) at eight weeks of age and maintained under a specific pathogen-free condition with controlled temperature and humidity and a 12-h light / 12-h dark cycle. They were given standard laboratory diet and water ad libitum. All animal procedures were performed in accordance with the Guidelines for Animal Research at Jichi Medical University (Tochigi) and approved by the Use and Care of Experimental Animals Committee.

In the chronic study, rats (n = 16) were divided into the following four groups (n = 4 in each): Group I (control group) received i.p. injection of saline (5 $ml \cdot kg^{-1} \cdot day^{-1}$) and gavage administration of 0.5% carboxymethyl cellulose sodium salt (CMC) solution as a vehicle; Group II (ATO group) and Group III (ATO + LA group) received i.p. injection of ATO (5 $mg \cdot kg^{-1} \cdot day^{-1}$) and gavage administration of vehicle or LA (35

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$mg \cdot kg^{-1} \cdot day^{-1}$), respectively; and Group IV (LA group) received i.p. injection of saline and oral administration of LA (35 $mg \cdot kg^{-1} \cdot day^{-1}$). After 8 weeks of treatment, the 24-h urine sample was collected after the last dosing. Blood samples were also obtained.

In the acute study, rats (n = 32) were anesthetized with i.p. injection of urethane (1.3 g/kg), and the right femoral vein was catheterized for drug infusion. The lead I ECG was continuously recorded by an ECG recorder (PowerLab; AD Instruments, Colorado Springs, CO, USA) from several minutes before drug injection as follows (n = 4 in each group): 1) saline alone; 2) 0.15 mg/kg ATO, a dose commonly used in humans (9, 10); 3) 1.5 mg/kg ATO; and 4) 5 mg/kg ATO. Effect of LA on the ATO-related ECG abnormality was also evaluated as follows (n = 4 in each group): 1) Control group: infusions of saline, twice; 2) ATO group: infusions of saline and ATO (5 mg/kg); 3) ATO + LA group: infusions of LA (70 mg/kg) and ATO (5 mg/kg); and 4) LA group: infusions of LA (70 mg/kg) and saline. ATO was given at 10 min after LA dosing. The same volume of saline was used in each infusion. The dose of LA (70 mg/kg) was selected by reference to a previous study (11) reporting that a single intravenous injection of LA produced an acute protective effect on oxidative stress in the heart.

The ECG parameters were measured during the 2-h period (immediately before and at 10, 20, 30, 60, 90, and 120 min after infusion). The corrected QT interval (QTc) was calculated with the Bazett formula and the Waterfall Plot (three-dimensional image of the stacked averaged ECGs) was created using the ECG Analysis tool (MLS360, AD Instruments).

Serum concentrations of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and creatinine (sCr) and urinary concentration of 8-hydroxy-2′-deoxyguanosine (8-OHDG), a marker of oxidative stress, were assayed using commercial kits.

All data are presented as the mean ± S.E.M. One-way ANOVA was used to compare means among groups. The Bonferroni/Dunn procedure was used as a post-hoc test. All statistical analyses were carried out with StatView 5.0 software (SAS Institute, Cary, NC, USA). Differences were considered to be significant at $P < 0.05$.

To determine the characteristics of the ATO-related adverse effects and potential preventive effect of LA, ATO, and LA were repeatedly given to Wistar rats for eight weeks. As a result, two of the four ATO-treated rats suddenly died without prior symptoms of infection and cerebrovascular disease on days 25 and 28, respectively. However, no rats died in the other three groups. ATO reduced the body weight and serum AST concentration and increased the urinary excretion of 8-OHDG in the surviving rats (Table 1). Interestingly, LA did not ameliorate these ATO-related changes (Table 1), although the agent prevented the ATO-induced death in this study. Therefore, the cause of death in rats with ATO alone might not be malnutrition, chronic organ damage, or bone marrow suppression.

LA is a potent biological antioxidant and is reported to scavenge free radicals, chelate metals, and restore intracellular glutathione level (12). We previously showed that ATO causes renal cell damage with the elevation of heme oxygenase 1, which is induced by oxidative stress, and LA ameliorates the ATO-induced cytotoxicity by reducing superoxide anion production (7). These data led us to speculate that the protective effect of LA against the

Table 1. Effects of ATO and LA on the laboratory parameters

Parameter	Control	ATO[§]	ATO + LA	LA
Body weight (g)	267.1 (245.5, 286.2)	231.8 (217.5, 243.9)	223.5* (205.4, 242.0)	253.1 (249.0, 257.6)
AST (IU/mL)	88 (83, 91)	66 (58, 74)	59* (50, 78)	81 (68, 93)
ALT (IU/mL)	36 (32, 38)	31 (28, 34)	38 (32, 53)	40 (32, 48)
ALP (IU/mL)	707 (649, 779)	598 (547, 649)	547 (444, 610)	669 (582, 848)
sCr (mg/dL)	0.29 (0.27, 0.33)	0.33 (0.27, 0.39)	0.27 (0.27, 0.27)	0.27 (0.25, 0.28)
8-OHDG (ng/day)	261.7 (177.9, 332.0)	462.7 (252.8, 672.6)	447.6* (340.6, 535.8)	307.0 (270.9, 369.0)

Values are reported as the mean (min, max) (n = 4, [§]n = 2). *$P < 0.01$, compared to the control. Differences among three groups except for the ATO group were determined by ANOVA. ATO dosing reduced the body weight and serum AST concentration and increased the urinary excretion of 8-OHDG in the surviving rats.

ATO-induced adverse effect resides in its antioxidative effect. However, to our surprise, LA did not diminish the systemic ATO-induced oxidative stress, which was reflected in urinary 8-OHDG excretion. Therefore, it remains probable that LA prevents sudden death by a mechanism other than the protection against oxidative stress–associated organ damages.

Because ATO treatment can lead to QT prolongation and T-wave changes in clinical practice (13), we speculated that 1) ATO caused sudden death due to cardiac toxicities and 2) LA prevented ECG abnormalities induced by ATO in rats. Then, we examined whether ATO induces abnormal ECG findings. The previous study showed that intravenous ATO (0.15 and 1.5 mg/kg) acutely prolonged QT interval in guinea pigs (14). However, infusion of the same (0.15 and 1.5 mg/kg) and higher (5 mg/kg) doses of ATO in Wistar rats did not change the QT interval (QTc) in this study (data not shown). However, the higher dose (5 mg/kg) of ATO caused transient ST-T wave changes on the ECG, from approximately 5 – 30 min after infusion (Fig. 1). In addition, ATO treatment significantly ($P < 0.01$) prolonged PQ interval by about 6 ms from 10 to 120 min after infusion (Fig. 1). Next, we examined the effect of LA pretreatment on such ECG abnormalities and found that both the ST-T wave change and PQ-interval prolongation induced by ATO treatment were completely prevented by co-administration of LA (Fig. 2). No other abnormal ECG findings, such as prolonged intervals of QT/QTc, PR, and QRS, were detected in the ATO or LA groups (Fig. 2).

The present study demonstrated for the first time that LA exerts a protective effect against the ATO-induced ST-T wave change and PQ-interval prolongation. Considering that ATO affects at least cardiac potassium currents (15), LA seems to prevent the ATO-induced electrical abnormalities. Because dithiol chelating compounds, such as DL-2,3-dimercaptopropanol (British Anti-Lewisite, BAL), DL-2,3-dimercaptopropanesulfonate (DMPS), and meso-2,3-dimercaptosuccinic acid (DMSA), are effective in reducing acute ATO poisoning by reacting with trivalent arsenic (16), it is possible that LA might reduce the ATO-induced acute cardiac toxicity via the same mechanism. Further studies are needed to reveal the underlying mechanism of the protective effect of the agent.

The dose of ATO that caused ECG abnormalities in rats was about 30 times higher than the therapeutic dose in the usual clinical setting. However, a phase I/II clinical trial provided data showing the long elimination half-life (approximately 100 h) of arsenic and gradual elevation in blood concentration of the agent during the repeated dosing (Product information from Nippon Shinyaku, 2010). Plasma arsenic concentration is reported to elevate to 2 to 5 times higher concentrations at one week after the initiation of treatment. Therefore, it is possible that

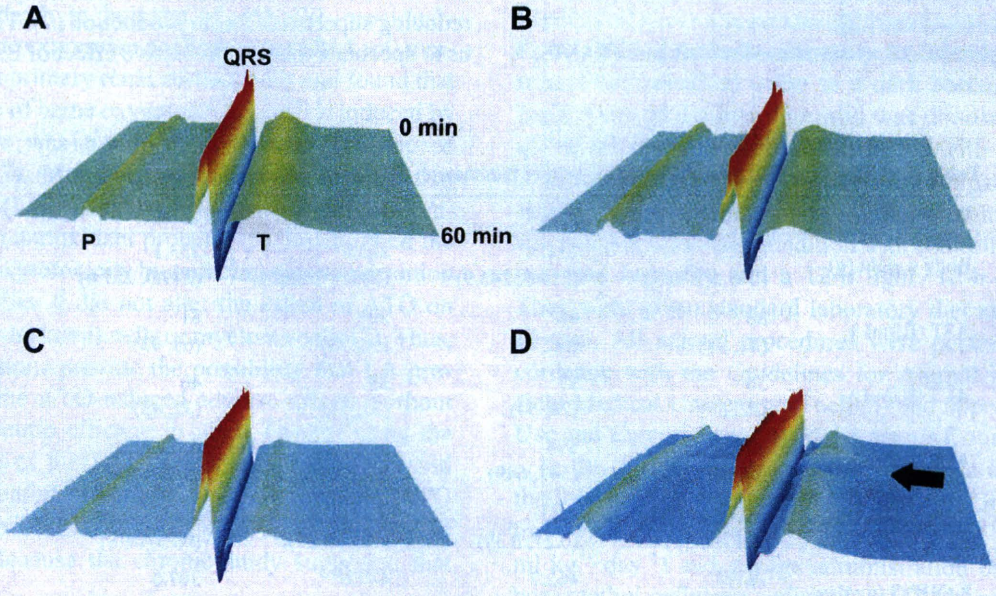

Fig. 1. Changes of ECG waveform after the intravenous infusion of ATO in rats. Representative three-dimensional images of the stacked averaged ECGs in rats treated with saline (A) or 0.15 (B), 1.5 (C), and 5 (D) mg/kg of ATO are presented. Continuous ECG monitoring revealed that only 5 mg/kg of ATO caused a transient ST-T change (arrow) from approximately 5 to 30 min after infusion.

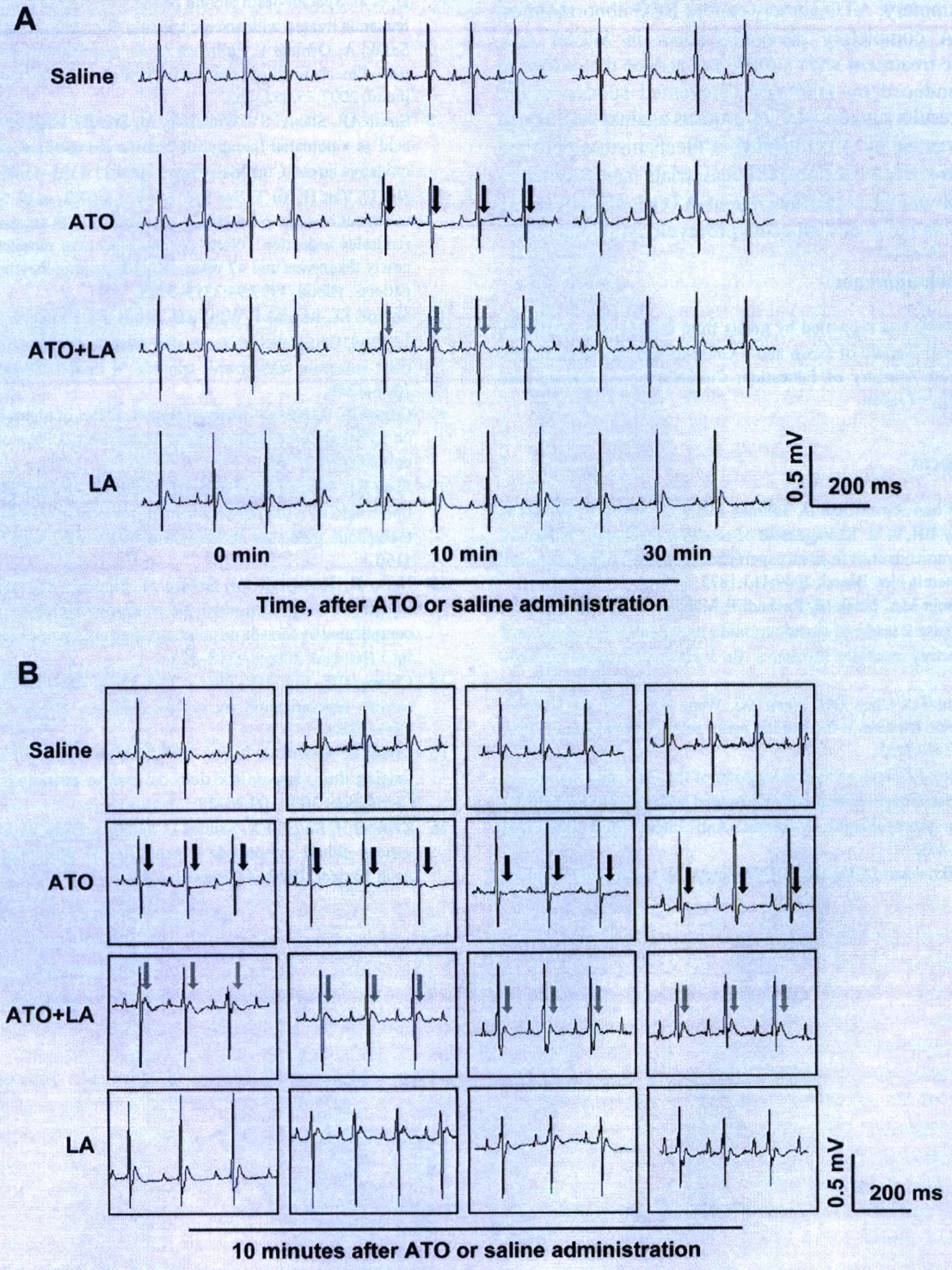

Fig. 2. Changes of ECG waveform after intravenous infusion of ATO in rats pretreated with and without LA. A) Lead I ECG waveforms at 0, 10, and 30 min after saline or ATO infusion in one representative rat. B) Lead I ECG waveforms at 10 min in all animals. The ST-T change induced by 5 mg/kg of ATO alone was reproducibly detected in all animals (black arrows), and the pretreatment with LA completely abolished such an ECG abnormality (gray arrows).

the arsenic concentration after multiple doses of ATO was high enough to cause myocardial toxicity during the repeated treatment in the first study.

In summary, ATO acutely caused ECG abnormalities, and LA completely prevented them in Wistar rats. Chronic treatment with LA did not reduce the oxidative stress induced by ATO, but prevented sudden death. These results suggest that LA protects against the adverse effect caused by ATO through a mechanism other than the anti-oxidant action. Clinical trials are needed to confirm whether LA can prevent ATO-induced cardiac toxicity in patients with acute promyelocytic leukemia.

Acknowledgments

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Inhibition of heat shock protein 90 attenuates adenylate cyclase sensitization after chronic morphine treatment

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ABSTRACT

Cellular adaptations to chronic opioid treatment result in enhanced responsiveness of adenylate cyclase and an increase in forskolin- or agonist-stimulated cAMP production. It is, however, not known whether chaperone molecules such as heat shock proteins contribute to this adenylate cyclase sensitization. Here, we report that treatment of cells with geldanamycin, an inhibitor of heat shock protein 90 (Hsp90), led to effective attenuation of morphine-induced adenylate cyclase sensitization. In SK-N-SH human neuroblastoma cells, morphine significantly increased RNA transcript and protein levels of type I adenylate cyclase, leading to sensitization. Whole-genome tiling array analysis revealed that cAMP response element-binding protein, an important mediator for cellular adaptation to morphine, associated with the proximal promoter of Hsp90AB1 not only in SK-N-SH cells but also in rat PC12 and human embryonic kidney cells. Hsp90AB1 transcript and protein levels increased significantly during morphine treatment, and co-application of geldanamycin (0.1–10 nM) effectively suppressed the increase in forskolin-activated adenylate cyclase activation by 56%. Type I adenylate cyclase, but not Hsp90AB1, underwent significant degradation during geldanamycin treatment. These results indicate that Hsp90 is a new pharmacological target for the suppression of adenylate cyclase sensitization induced by chronic morphine treatment.
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1. Introduction

Chronic stimulation of cells by an opioid agonist causes opioid dependence as an adaptation process [1]. One of the biochemical changes during chronic opioid treatment is a significant increase in adenylate cyclase activities stimulated by forskolin or Gs protein-coupled receptor [2,3]. This enhanced responsiveness, known as adenylate cyclase superactivation, eventually counteracts the acute effect of the Gi/o-coupled opioid receptor in inhibiting adenylate cyclase [2–5]. Of three opioid receptor subtypes, μ-opioid receptor (MOR) is the primary target for morphine analgesia. Chronic stimulation of MOR by morphine enhances intracellular cAMP signaling and eventually alters gene transcription mediated by the transcription factor cAMP response element-binding protein (CREB) [6]. Recent DNA microarray technology has enabled the extraction of genes from the entire genome whose expression is altered by morphine treatment [7–12] and of gene promoters that physically associate with CREB [13–16]. Because CREB receives multiple signaling inputs in addition to cAMP signaling and regulates transcription of a wide range of genes, the identification of unique pharmacological targets has been a challenging task.

Heat shock proteins (Hsps) constitute a large family of highly conserved proteins and are categorized according to their molecular weights. Hsps protect cells from cellular stress and promote adaptation to stress conditions by preventing protein aggregation and facilitating the refolding of denatured proteins [17,18]. Although enhanced expression of several Hsps by morphine has been reported [19], the functional significance of these molecular chaperones in morphine dependence has not been defined.

In this study, we searched for a new drug target to modulate adenylate cyclase superactivation in a SK-N-SH human

Abbreviations: TG, [Thr[4]-Gly[7]]-oxytocin; Mpomeovt, 1-deamino-2-O-methyl-tyrosyl-8-ornithine-1-(beta-mercapto-(beta,beta-cyclopentamethylene)propionic acid)oxytocin; Hsp90, heat shock protein 90; MOR, μ-opioid receptor; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; OT, oxytocin; ATII, angiotensin II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation.

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neuroblastoma cell line that natively expresses MOR. We found that CREB associates with the promoter of the heat shock protein 90 AB1 (Hsp90AB1) gene and that Hsp90AB1 expression is enhanced by chronic morphine treatment. Furthermore, an Hsp90 inhibitor was found to significantly suppress adenylate cyclase superactivation, thereby providing a new strategy for effective manipulation of the cellular model of opioid dependence.

2. Materials and methods

Cell culture. SK-N-SH human neuroblastoma cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Adenylate cyclase assay. A sample of 10^6 cells in a 10-cm tissue-culture dish were stimulated with saline, morphine, oxytocin (OT), or angiotensin II (ATII) for 24–72 h. Cells were washed twice with PBS, collected in PBS containing 1 mM EDTA (pH 8.0), centrifuged at 1000g for 2 min, and resuspended in buffer A (DMEM containing 10 mM HEPES (pH 7.4) and 500 μM isobutyl methylxanthine) at 10^5 cells/ml. After incubation in buffer A at 37 °C for 30 min, 10^5 cells/well were stimulated for 5 min at 37 °C in the presence or absence of forskolin. The reaction was terminated by heating at 100 °C for 5 min, and cellular supernatants were stored at −20 °C until the cAMP assay. For the determination of cAMP production, 10 μl of the supernatant was subjected to a non-radioactive enzyme immunoassay kit (PerkinElmer, MA). To evaluate adenylate cyclase inhibition, cAMP concentrations were converted to a percentage of the maximum response obtained by a defined concentration of forskolin. An Hsp90 inhibitor, geldanamycin (GA), was obtained from Tokyo chemical industry Co. Ltd., and initially dissolved in dimethyl sulfoxide at 100 mM.

Intracellular calcium measurement. Cells were incubated at 37 °C for 60 min with 1 μM fluo4-AM (Invitrogen, CA) in an assay buffer containing 137 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), and 10 mM glucose. The cells were subsequently washed two times with the assay buffer. The intracellular calcium-ion concentration ($[Ca^{2+}]_i$) responses were examined under a 60× oil-immersion objective using a confocal microscope (FV300, Olympus, Japan). The intensity of light emission at 520 nm was measured at frequency of 1 Hz for 3 min. The series of images obtained were analyzed using the ImageJ software program (Version 1.43b, NIH, USA).

Real-time PCR assay. The amounts of expressed transcripts were examined by real-time quantitative PCR (qPCR) as described previously [20]. The results were normalized by expression of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR primer sequences were selected using the BatchPrimer3 software package [21] and are listed in Supplementary Table 1.

Western blot analysis. Antibody specificity to CREB (Millipore, MA) was examined by Western blot analysis as described previously [22]. Peroxidase-conjugated anti-rabbit antibody (GE Healthcare UK Ltd., Amersham Place, UK) was used at a dilution of 1:5000, antibodies to Hsp90AB1 (Abcam Corp. Japan) were used at a dilution of 1:5000, and antibodies to adenylate cyclase type I (Santa Cruz Biotech, CA) were used at a dilution of 1:200. Signals were visualized with enhanced chemiluminescence ECL (GE Healthcare UK Ltd.).

Chromatin immunoprecipitation and tiling array analysis. For chromatin immunoprecipitation (ChIP), 10^8 cells grown on 15-cm culture dishes were incubated in 1% formaldehyde for 10 min at ambient temperature and for a further 10 min in 125 mM glycine before harvesting. After being rinsed three times with PBS, the cell pellets were washed three times with a lysis buffer (10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% IGEPAL, and 1 mM phenylmethylsulfonyl fluoride). Immunoprecipitation of CREB, the labeling procedure, and tiling array hybridization were performed according to the manufacturer's instructions (GeneChip® Human Tiling 2.0R Array Set, Affymetrix, Santa Clara, CA). The CEL file data obtained from two biological replications were subjected to analysis with model-based tiling array analysis software (MAT) for the detection of a ChIP-enriched genomic locus with a BPMAP file prepared for hg18 human genome assembly (National Center for Biotechnology Information Build 36) [23]. The *P* value for the cutoff of the MAT program was set at 10^{-6}. The Integrated Genome Browser (Affymetrix) and UCSC website were used [24] to visualize chromosomal locations in the human and rat genome (hg19 and Rnor3.4, the Rat Genome Sequencing Consortium). For the analysis of genomic intervals, R statistical environment (version 2.9.2) was used [25].

Statistics. Significant differences were determined by either Student's *t*-tests or one-way analysis of variance (ANOVA) tests followed by Holm's tests, if applicable, and a *P* value of <0.05 was considered to represent a significant difference. The concentration–response curves were fitted to a four-parameter logistic function by using a non-linear curve-fitting program (Igor Pro 6.04, WaveMetrics, Lake Oswego, OR).

3. Results

Adenylate cyclase superactivation in a cellular model for opioid adaptation

We used a SK-N-SH human neuroblastoma cell line to produce a cellular model of chronic morphine treatment. When the cells were incubated with 10 μM morphine or 0.1 μM oxytocin for 48 h, cAMP production by forskolin was significantly enhanced compared to the control cells or cells treated with 0.1 μM ATII (Fig. 1A). Real-time PCR analysis of the transcripts in the cells detected the MOR as the major opioid receptor subtype (transcript amounts normalized by those of β-actin were 1.3 ± 0.3 and $0.15 \pm 0.01 \times 10^{-2}$, for the μ- and δ- receptor subtypes, respectively; $P < 0.05$, $n = 3$). The transcript for the κ-opioid receptor was not detected. In SK-N-SH cells, the OT receptor is mainly expressed among the receptors for OT/arginine-vasopressin (AVP) peptides (transcript amount normalized by those of β-actin were 2.6 ± 0.2, 0.19 ± 0.03, 0.08 ± 0.01, and $0.04 \pm 0.01 \times 10^{-2}$ for OT, V1a, V1b, and V2 receptors, respectively; $n = 3$). Stimulation of the cells with an OT-specific agonist, [Thr^4-Gly^7]-oxytocin (TG), caused a transient increase in $[Ca^{2+}]_i$, and following AVP (1 μM) did not change $[Ca^{2+}]_i$, because of OT receptor desensitization (Fig. 1B). The amplitude of $[Ca^{2+}]_i$ response was significantly reduced by pre-incubation with an OT antagonist, Mpomeovt (Fig. 1B). OT and AVP instantly inhibited forskolin-induced cAMP production in this cell line (Fig. 1C). IC_{50} values for the inhibition of adenylate cyclase activity stimulated by 1 μM forskolin were 3.4 and 22 nM for OT and AVP, respectively, $n = 4$. Co-application of both morphine and OT enhanced the inhibitory capacity compared to that of morphine alone (Fig. 1D). These results indicate that the SK-N-SH cell line is an appropriate model for the study of adenylate cyclase superactivation induced by chronic activation of Gi-coupled receptors.

Analysis of CREB binding sites by the ChIP-Chip method

Because CREB has been implicated as a key transcription factor in developing and maintaining addiction to narcotic drugs [26], we analyzed its binding sites to the human genome in SK-N-SH cells. Specificity of CREB antibody was confirmed in a Western blot analysis; the expected signal with electrophoresis mobility corre-

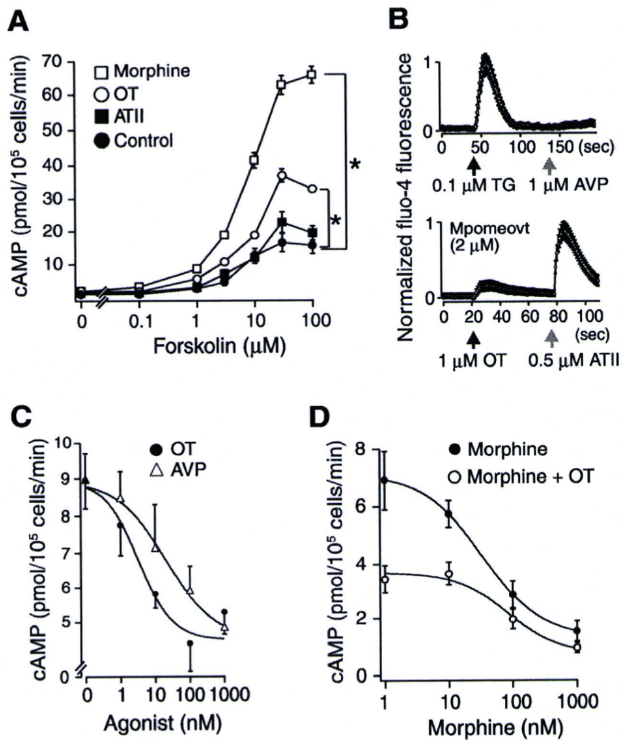

Fig. 1. Adenylate cyclase superactivation in SK-N-SH cells. (A) The cells were treated with saline, 10 μM morphine, 0.1 μM OT, or 0.1 μM ATII for 48 h, washed two times, and incubated at 37 °C for 30 min to remove bound ligands. Basal and forskolin-stimulated cAMP production was then measured as described in Material and methods. The figure shows results from six experiments performed in triplicate. *$P < 0.05$ vs. saline. (B) $[Ca^{2+}]_i$ responses were evoked by an OT-receptor selective agonist, [Thr^4-Gly^7]-oxytocin (TG) (upper panel). Pre-incubation of the cells with 2 μM Mpomeovt, an OT-receptor antagonist, for 5 min effectively inhibited the $[Ca^{2+}]_i$ response by OT, but not by ATII (lower panel). Each point of these $[Ca^{2+}]_i$ responses represents mean ± SD from 10 to 12 cells measured simultaneously. The figures presented are representative from three independent $[Ca^{2+}]_i$ measurements performed in triplicate dishes. (C) The OT receptor was also coupled to adenylate cyclase inhibition ($n = 5$). Cells were stimulated with 1 μM forskolin in the presence of different concentrations of AVP (open triangle) or OT (closed circle). (D) Co-application of both morphine and OT enhanced inhibition of forskolin-induced cAMP production. Cells were stimulated with 1 μM forskolin in the presence of different doses of morphine (filled circle) or a combination of morphine and 0.1 μM OT (open circle) at 37 °C for 5 min. The figure shows results from four experiments performed in triplicate.

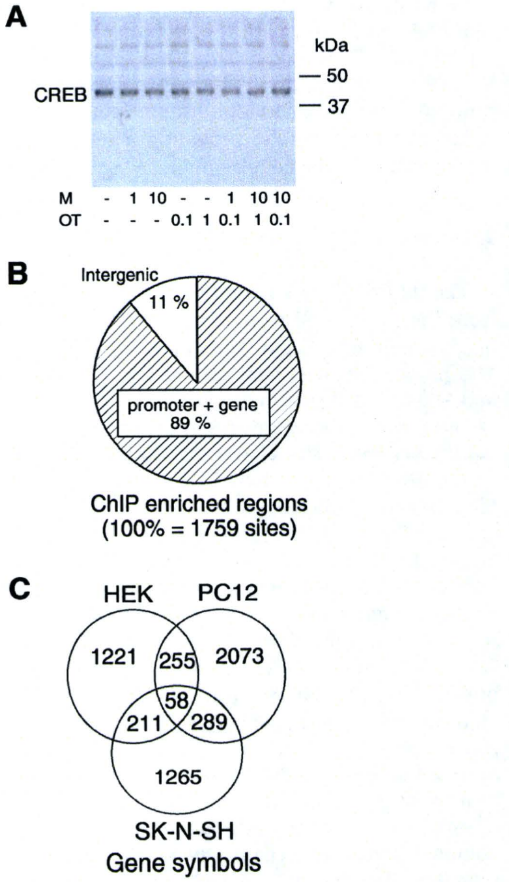

Fig. 2. ChIP-Chip analysis of CREB binding sites in SK-N-SH cells. (A) Cells treated with morphine (M) and/or OT were subjected to a Western blot analysis. A specific signal of ~43 kDa corresponding to CREB was detected. Application of morphine and OT did not change the total amount of CREB. (B) CREB-associated genomic regions from microarray data were mapped to a human genome assembly (hg19) and nearby gene loci were identified. A CREB-associated gene was defined if a RefSeq gene or promoter region 3.5 kb upstream from the transcriptional initiation site includes sequences of genomic fragments precipitated by anti-CREB antibodies. (C) Conservation of CREB-associated genes was analyzed in three distinct cell lines: SK-N-SH, HEK293T, and PC12. Gene symbols of the CREB-associated genes were compared, and a Venn graph was plotted. Data for the HEK293T and PC12 cell lines are from previous whole-genome studies [14,15].

sponding to 43 kDa was detected (Fig. 2A). By using the ChIP-Chip method [14], precipitated genomic DNA fragments with anti-CREB antibody were hybridized to a tiling array covering the entire non-repetitive human genome. In morphine-sensitive SK-N-H cell, the 1759 genomic regions physically associated with CREB, and a majority of the CREB-associated genomic regions (89%) were located within or near gene loci found in RefSeq data base (Fig. 2B). When conservation of the CREB-associated gene profiles was examined in neuroblastoma-derived SK-N-SH cells, embryonic kidney HEK293T cells, and rat PC12 pheochromocytoma cells, we found that 58 genes, corresponding to 3.2% of all CREB-associated genes in SK-N-SH cells, are commonly associated with CREB in all three cell types (Fig. 2C and Supplementary Table 2).

CREB is associated with the Hsp90AB1 gene, and expression of Hsp90AB1 was increased by morphine treatment

From the list of 58 genes that were commonly associated with CREB, we further examined the Hsp90AB1 gene because Hsp90 inhibitors are available and are under development in clinical trials for the treatment of cancer patients [27]. As shown in Fig. 3A, association of CREB with the promoter immediately upstream of the Hsp90AB1 heat shock protein gene, but not with the β-actin gene locus, was confirmed by ChIP-PCR. Interestingly, the enriched DNA was significantly decreased after morphine treatment (64% ± 1% of control, $n = 3$, Fig. 3A). Furthermore, Hsp90AB1 transcripts and protein levels were significantly increased by chronic morphine treatment (Fig. 3B and C).

Geldanamycin, a Hsp90AB1 inhibitor, has an inhibitory effect on adenylate cyclase superactivation induced by chronic morphine

A real-time qPCR experiment found that among the nine adenylate cyclase subtypes, only type I adenylate cyclase (ADCY1) expression was increased after 48 h of treatment with morphine compared to control cells (1.6 ± 0.2-fold increase, $P < 0.05$, $n = 3$). As shown in Fig. 4A, ADCY1 protein level also significantly increased after chronic morphine treatment. In contrast, GA dose-dependently reduced the protein levels of ADCY1 but not Hsp90AB1 (Fig. 4B). Moreover, incubation of GA with morphine inhibited