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PAPER

PATHOLOGY/BIOLOGY

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Increased Heat Shock Protein 70 Gene Expression in the Brains of Cocaine-Related Fatalities may be Reflective of Postdrug Survival and Intervention rather than Excited Delirium

ABSTRACT: Cocaine-related fatalities can pose forensic challenges, particularly when accompanied by excited delirium (ED) syndrome and interventions by law enforcement and medical personnel. A recent report concluded that elevated heat shock protein 70 (HSP70) expression in autopsy brain samples constitutes a reliable forensic biomarker for the identification of ED as a cause of death. The present study quantified the abundance of both *HSPA1A* and *HSPA1B* gene (HSP70-encoding) transcripts in midbrain specimens from a series of cocaine-related fatalities and matched drug-free control subjects. HSP70 expression was increased significantly in cocaine abusers as a group compared to control subjects, irrespective of the presence or absence of ED. Furthermore, elevated HSP70 expression was predictive of a period of survival between cocaine use and death that included medical and/or police intervention. The present data do not support the assertion that HSP70 expression is a reliable brain biomarker for identifying ED as a cause of death.

KEYWORDS: forensic science, forensic pathology, excited delirium, heat shock protein 70, cocaine, survival time, autopsy, human brain, microarray

Deaths associated with cocaine abuse can pose forensic challenges, particularly when sudden unexplained death follows the emergence of excited delirium (ED), a syndrome characterized by agitation, combativeness, and hyperthermia. These subjects often die in custody following confrontations with police that involve physical struggle, restraint, and/or deployment of conductive energy devices (for reviews, see [1,2]. A recent study (3) reported that heat shock protein 70 (HSP70) expression was increased in the postmortem brains of cocaine subjects exhibiting ED in comparison with other (non-ED) cocaine-related deaths and drug-free controls, concluding that elevated HSP70 provides a reliable forensic biomarker for the identification of ED. As part of our broader effort to determine the profile of gene expression in cocaine abusers' brains (4–8), we examined HSP70-related gene expression in postmortem specimens from a series of cocaine-related deaths and well-matched drug-free control subjects. *HSPA1A* and *HSPA1B* (i.e., HSP70-encoding) gene expression was significantly increased in the brains of cocaine abusers as a group compared to drug-free controls, irrespective of the presence or absence of ED. Furthermore, elevated HSP70 expression was significantly predictive of a period of survival between cocaine use and death that included medical and/or police intervention. The previous assertion that HSP70 constitutes a reliable forensic tool for the neuropathological identification of ED as a cause of death is discussed in light of these findings.

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Materials and Methods

Case Acquisition and Characterization

Postmortem ventral midbrain specimens were obtained at autopsy and analyzed as described previously (4–8). Briefly, the cause and manner of death were determined by forensic pathologists after medico-legal investigations that evaluated the circumstances of death including police reports and medical records, autopsy results, and toxicological data. All cases were evaluated for common drugs of abuse (including alcohol), and positive urine screens were confirmed by quantitative analysis of blood levels of cocaine and metabolites (9). The cocaine abuse cohort in the present study had a documented history of drug abuse, tested positive for cocaine and/or metabolites but negative for other drugs of abuse, and were determined to have died as a result of cocaine abuse/cocaine intoxication. Two of the cocaine-related fatalities also exhibited ED syndrome (see Table 1), as evidenced by the emergence of abnormal behaviors immediately following cocaine

TABLE 1—*Demographic characteristics and forensic data on cocaine fatalities and matched control cases.*

Case	Age	Sex/ Race*	COD**	Cocaine (ug/mL)	Benzoylecgonine (ug/mL)	Survival Time (h)	Case	Age	Sex/ Race	COD	Cocaine (ug/mL)	Benzoylecgonine (ug/mL)	Survival Time (h)
#27	51	BF	ASCVD	ND†	ND	0	#28	52	BF	Cocaine, cerebral hemorrhage	ND	0.077	0
#33	30	WF	GSW	ND	ND	0	#34	35	WF	Cocaine abuse	0.28	4.4	0
#39	20	WM	Dilated cardiomyopathy	ND	ND	0	#40	25	WM	Cocaine	13	7.4	0
#1	40	BF	Dilated cardiomyopathy	ND	ND	0	#2	34	BF	Cocaine abuse, cardiac arrest	ND	0.66	0
#7	47	WM	Acute MI, ASCVD	ND	ND	0	#8	46	WM	Acute cocaine intoxication	0.8	5.7	0
#5	35	BM	GSW (ASCVD contributing)	ND	ND	4	#6	35	BM	Acute cocaine intoxication	31	8.9	0
#9	45	BM	MGSW	ND	ND	0	#10	46	BM	Cocaine abuse	0.069	1.5	0
#11	49	BM	MI, ASCVD	ND	ND	0	#12	49	BM	Aortic aneurysm owing to cocaine abuse	ND	0.42	0
#50	53	BM	ASCVD	ND	ND	0	#51	59	BM	Cocaine abuse	ND	0.071	0
#52	51	BM	Aortic dissection, hypertension	ND	ND	0	#53	54	BM	Cocaine abuse	ND	0.09	0
#54	66	BM	MGSW	ND	ND	0	#55	64	BM	Acute cocaine intoxication	0.065	0.4	0
#56	46	BM	MGSW	ND	ND	0	#57	52	BM	Cocaine abuse	0.078	0.33	0
#41	45	WM	GSW	ND	ND	0	#42	46	WM	Cocaine abuse‡	ND	0.03	1
#3	33	BM	Dilated cardiomyopathy	ND	ND	0	#4	35	BM	Cocaine abuse‡	14	8.1	1
#35	34	BM	MGSW	ND	ND	0	#36	34	BM	Cocaine abuse	0.74	4.3	1
#15	52	BM	Hypertensive cardiomyopathy	ND	ND	0	#16	52	BM	Aortic dissection owing to cocaine abuse	ND	0.58	3
#13	50	BM	GSW	ND	ND	0	#14	52	BM	Cocaine abuse	0.29	3	5
#37	45	BM	ASCVD	ND	ND	0	#38	45	BM	Cocaine abuse	0.053	3.4	21

Cases were analyzed groupwise but listed pairwise for ease of comparison.

Control and cocaine groups did not differ in terms of age (44 ± 3 and 45 ± 2, respectively) or sex/race (2 BF, 1 WF, 3 WM, 12 BM in each group).

*BF, black female; WF, white female; WM, white male; BM, black male.

**COD, cause of death; ASCVD, arteriosclerotic cardiovascular disease; GSW, gunshot wound; MGSW, multiple gunshot wounds; MI, myocardial infarction.

†ND, not detected.

‡Cases exhibited abnormal behaviors clearly indicative of ED syndrome.

ingestion, followed by contact with medical and/or law enforcement personnel. One of the authors (a trained forensic pathologist) reviewed the case files of cocaine-related fatalities before final classification and inclusion of cases. Control subjects had no documented history of drug abuse, were drug-free at the time of death (with the exception of a single control with a sub-intoxicating level of alcohol), and died as a result of cardiovascular disease or gunshot wounds. Cocaine abusers and drug-free controls were carefully matched for age, race, gender, and sample quality measurements including brain pH and RNA integrity number (RIN) and added pairwise to the study for a total of $n = 18$ in each group. Subject demographics, cause of death, blood levels of cocaine and metabolites, and estimated survival time between initial contact with police and/or medical personnel and death are summarized in Table 1. Drug abuse and control groups did not differ in age, race, or gender (Table 1). Nor did the control and cocaine groups differ in terms of the postmortem sample quality indicators of brain pH (6.5 ± 0.05 and 6.5 ± 0.05, respectively) or RIN (6.5 ± 0.2 vs. 6.7 ± 0.2, respectively). All data reported as means ± SEM.

Specimen Processing, Microarray, and RT-PCR

Ventral midbrain sample processing and subsequent gene expression analysis by microarray and quantitative real-time PCR (qRT-PCR) were implemented as recently described in detail (10). Briefly, fresh-frozen midbrain blocks were cut at a thickness of 250 μm on a cryostat and slide-mounted for subsequent hand dissection of dopamine cell-enriched regions of the ventral midbrain. Other specified brain regions were dissected free of surrounding tissue at the time of autopsy and frozen for subsequent use. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and then DNAse-treated with the Qiagen RNeasy Minikit (Qiagen, Valencia, CA). Quantification of RNA was accomplished using NanoDrop ND-1000 (Thermo-Scientific, Waltham, MA), with an initial assessment of RNA quality by agarose–formaldehyde gel electrophoresis and ethidium bromide staining.

Microarray assays (using HT-12 BeadChips; Illumina, Inc., San Diego, CA) were performed by the Keck Microarray Resource as part of the NIH Neuroscience Microarray Consortium (Yale Center for Genomic Analysis, West Haven, CT). The quality and quantity of each RNA sample was verified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) prior to labeling reactions. Biotin-labeled cRNAs were generated using the TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, CA) with 500 ng total RNA as template. Each sample was labeled in an independent reaction, using technical triplicates to obtain a measure of experimental variation. In addition, an RNA pool derived from multiple samples was hybridized across arrays to assess potential interarray variation. Microarray controls included control RNAs spiked into RT reactions, as well as spiked-in cRNAs that matched or

mismatched with BeadChip oligos. Labeled cRNAs were hybridized according to the manufacturer's protocol and scanned on the Illumina Iscan. Initial Consortium data analysis included quantile normalization carried out in Illumina BeadStudio.

For qRT-PCR validation experiments, 100 ng RNA (aliquots of the same samples used for microarray experiments) from individual subjects was reverse-transcribed using random hexamers (Promega, Madison, WI) and Omniscript RT kit (Qiagen) following the manufacturer's protocol. Subsequent PCR used SYBR Green master mix (Qiagen) following the manufacturer's instructions. Transcript abundance in individual samples (assayed in duplicate) was quantified using the StepOne® Real-Time PCR System (Applied Biosystems) in comparison with a six-point standard curve generated from pooled sample aliquots. PCR primer sequences (represented 5′-3′) used in validation experiments were forward primer ACCATT GAGGAGGTAGATTAGG and reverse primer GCAAACACAGG AAATTGAGAAC for *HSPA1A*, and forward primer ACTGTTG GGACTCAAGGAC and reverse primer ATGAAGCCAGCTAAT TACCATC for *HSPA1B*.

Statistical Analysis

Normalized microarray data were imported into Microsoft Excel (Redmond, WA), and the statistical significance of differences in HSP70 between the cocaine and control groups was initially assessed using a groupwise *t*-test ($\alpha = 0.05$). The significance of differences between subgroups was assessed by one-way ANOVA in GraphPad Prism (Graphpad Software, Inc., La Jolla, CA) ($\alpha = 0.05$). Pearson's correlation coefficients (r) between log2-transformed microarray data and qRT-PCR data and between array data and cocaine/metabolite levels were calculated. The performance of HSP70 RNA abundance as a diagnostic test for subject survival was calculated by receiver operating characteristic curve analysis in SPSS (IBM, Armonk, NY) ($\alpha = 0.05$).

Results

Microarray analysis was conducted on samples of ventral midbrain obtained at autopsy from a group of chronic cocaine abusers and drug-free control subjects carefully matched in terms of demographics (age, race, and sex) and tissue sample quality (as reflected by brain pH and RIN values) (Table 1, Materials and Methods). Cocaine abusers as a group exhibited large (*c.* twofold) increases in the abundance of two HSP70-encoding transcripts, namely *HSPA1A* mRNA (7659 ± 1741 vs. 3668 ± 363) and *HSPA1B* mRNA (11,459 ± 2401 vs. 6108 ± 494) (Fig. 1*A*; two-tailed $p < 0.04$ for both transcripts). Significantly more variance in the abundance of these transcripts was evident in the cocaine cohort compared to the control subjects (Fig. 1*A*; *F*-test $p < 0.0001$). Two of the 18 cocaine-related cases also exhibited ED syndrome prior to death (Table 1), but *HSPA1A* and *HSPA1B* transcript levels for these cases fell well within the range of values seen for other (non-ED) cocaine-related fatalities (Fig. 1*A*). There was no significant correlation between *HSPA1A* or *HSPA1B* transcript levels and blood levels of cocaine or its metabolites (data not shown). It was noted, however, that *HSPA1A* and *HSPA1B* transcript levels were significantly higher in cocaine fatalities with a documented period of postdrug survival and contact with medical personnel and/or police before death, compared to the other cocaine-related fatalities or drug-free controls (Fig. 1*A,B*). In fact, elevated abundance of either HSP70-encoding transcript alone was significantly predictive as a diagnostic test for a period of postcocaine survival and intervention ($p < 0.03$; calculated by receiver operating characteristic curve analysis).

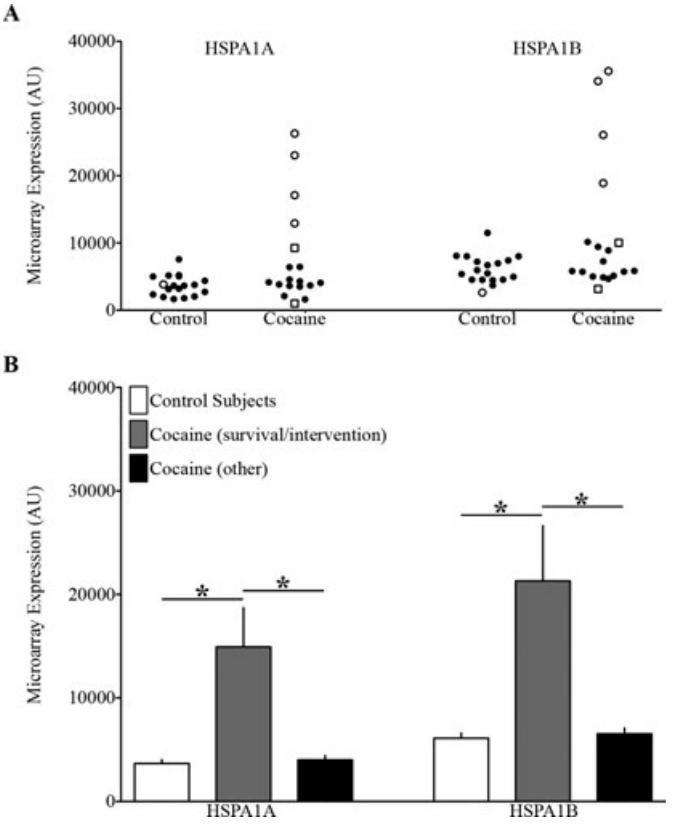

FIG. 1—*HSP70-related gene expression is elevated in postmortem midbrain specimens from cocaine-related fatalities compared to matched control subjects. HSPA1A and HSPA1B transcript abundance were determined by microarray analysis. (A) HSPA1A controls 3668 ± 363, cocaine 7659 ± 1741 (two-tailed* $p < 0.04$*). HSPA1B controls 6108 ± 494, cocaine 11,459 ± 2401 (two-tailed* $p < 0.04$*). Significance determined by groupwise* t*-test.* $n = 18$ *in each group. Filled circles indicate subjects declared dead at scene/on arrival. Open circles indicate some period of survival after contact with police/emergency personnel (for more information, see Table 1). Open squares indicate cocaine cases with ED syndrome. (B) The overall increase in HSP70 gene expression seen in cocaine fatalities is due to the subgroup with a documented period of postdrug survival and intervention (open circles above). Cocaine-related deaths after such a period of survival* ($n = 6$) *differed significantly from controls* ($n = 18$) *and cocaine fatalities without documented survival* ($n = 12$) *as determined by one-way ANOVA* ($p < 0.0001$) *followed by Tukey post-hoc tests. *Significant difference at* $\alpha = 0.05$.

Midbrain *HSPA1A* and *HSPA1B* transcript abundances as determined by microarray correlated significantly with subsequent qRT-PCR data (Pearson's r of 0.97 and 0.96, respectively; one-tailed $p < 0.0001$ for both transcripts; $n = 12$); therefore, qRT-PCR was used to assess HSP70 transcript abundance in several additional brain regions from four representative cases (i.e., a cocaine fatality found dead at the scene, a cocaine fatality exhibiting ED syndrome but short survival time, a cocaine fatality without ED but marked by prolonged survival time and medical interventions, and a drug-free control subject). As shown in Fig. 2, *HSPA1A* and *HSPA1B* transcript levels in ventral midbrain, caudate, nucleus accumbens, and corpus callosum were elevated in the cocaine fatality with prolonged postdrug survival and intervention in comparison with the other cocaine fatalities (either ED or non-ED) and the drug-free control (Fig. 2*A,B*), suggesting that some circumstances surrounding this death lead to a widespread induction of HSP70 gene expression throughout the central nervous system (CNS).

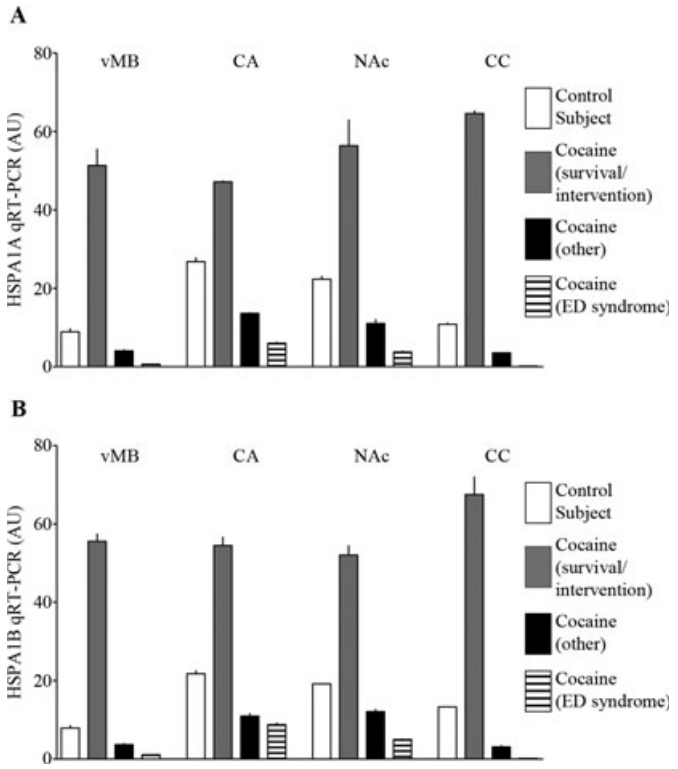

FIG. 2—*Individual differences in HSP70-related gene expression are seen across multiple brain regions. The abundance of HSPA1A (A) and HSPA1B (B) transcripts in ventral midbrain (vMB), caudate (CA), nucleus accumbens (NAc), and corpus collosum (CC) was determined by qRT-PCR in a representative cocaine fatality found dead at the scene, a cocaine fatality with ED syndrome and short survival time, a non-ED cocaine fatality marked by prolonged survival time and intervention, and a drug-free control subject.*

Discussion

Our ongoing effort to profile changes in gene expression in cocaine abusers' brains revealed unexpectedly large, statistically significant (though variable) changes in the abundance of HSP70-related *HSPA1A* and *HSPA1B* transcripts relative to well-matched drug-free control subjects. This finding was rather unexpected, given a report that *HSPA1B* transcript was selectively increased in the brains of ED cases relative to other (non-ED) cocaine-related fatalities and control subjects, leading to the proposal that increased HSP70 constitutes a specific forensic biomarker for ED (3). Although our conclusions about ED must be tempered by the small number of such cases involved, our study found that HSP70 gene expression was increased significantly in cocaine abusers as a group compared to drug-free controls, irrespective of the presence or absence of ED (Fig. 1). Unexpectedly, elevated HSP70 expression was instead predictive of a documented period of survival between cocaine use and death that included medical and/or police interventions, again irrespective of the emergence of ED syndrome (Fig. 1*B*).

Although our study focused on HSP70-related changes in the dopamine cell-rich ventral midbrain (Figs 1 and 2), a preliminary assessment of two cocaine-responsive forebrain regions (caudate nucleus and nucleus accumbens) as well as the corpus callosum (Fig. 2) suggests that cocaine-associated induction of HSP70 expression may be widespread in the CNS and thus does not account for the differences seen between our data and those of Mash et al. (3). The latter study focused on a large cohort of cocaine fatalities exhibiting ED, nearly all of whom experienced at least one force measure (e.g., mechanical restraints, conductive energy device, chemical agent) and most of whom survived 1–48 h after initial contact with police (3). Based on the present data, we hypothesize that the elevated HSP70 expression seen in the previous ED study (3) and also seen in a subset of our cocaine subjects is more likely to be related to postdrug survival time and/or interventions by medical and law enforcement personnel rather than the presence or absence of ED *per se*. In the current study, the single drug-free control subject with a prolonged survival time and medical interventions prior to death had *HSPA1A* and *HSPA1B* transcript levels that matched our other control cases (Table 1 and Fig. 1*A*), consistent with the notion that survival time and/or intervention(s) *after cocaine insult* may be key features in the induction of brain HSP70. Consistent with this interpretation, a previous forensic study has reported that the induction of HSP70 immunoreactivity in human hippocampus was associated with the length of survival after ischemic injury (11).

It is now better appreciated that HSP70 is rapidly induced in the CNS by a variety of cellular stresses (including hyperthermia and, in particular, ischemia), with its expression preceding, or even occurring in absence of, terminal physiological insult; HSP70 thus serves both biomarker and protective functions (for a review, see [12]). For example, overexpression of HSP70 protects midbrain dopamine neurons against cellular stresses and neurotoxins in animal models (13,14). Although human autopsy studies obviously cannot provide such causal data, the apparent association between elevated brain HSP70 expression and prolonged survival in cocaine-related fatalities could reflect an adaptive (although ultimately unsuccessful) response to limit cocaine-induced (perhaps ischemia-related) neurotoxicity.

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