Apoptotic Proteins in the Temporal Cortex in Schizophrenia: High Bax/Bcl-2 Ratio Without Caspase-3 Activation

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Objective: Neuroimaging findings have identified lower cortical gray matter volume in schizophrenia. Apoptosis (programmed cell death) has been proposed as a contributing pathophysiological mechanism. Levels of antiapoptotic Bcl-2 protein are low in the temporal cortex of schizophrenia patients. Bcl-2 interacts with the proapoptotic Bax protein at an upstream checkpoint to regulate the activation of apoptosis by caspase-3 and other proteolytic caspase proteins. A high Bax/Bcl-2 ratio is associated with greater vulnerability to apoptotic activation, while a high caspase-3 level is often associated with apoptotic activity. It was hypothesized that the Bax/Bcl-2 ratio, but not caspase-3, would be high in the temporal cortex of patients with chronic schizophrenia.

Method: Bax, Bcl-2, and caspase-3 proteins were measured by semiquantitative Western blot in Brodmann’s area 21 (middle temporal gyrus) of postmortem tissue from patients with schizophrenia (N=15), bipolar disorder (N=15), or major depression (N=15) and nonpsychiatric comparison subjects (N=15).

Results: The Bax/Bcl-2 ratio was 50% higher in the schizophrenia patients than the nonpsychiatric comparison subjects. The level of caspase-3 (inactive zymogen and activated subunits) was not significantly different.

Conclusions: The higher Bax/Bcl-2 ratio suggests that cortical cells are vulnerable to apoptosis in chronic schizophrenia. However, the normal caspase-3 level suggests that apoptosis is not active in this illness phase. Furthermore, the results appear to distinguish the pathophysiology of schizophrenia from most classic neurodegenerative disorders, in which postmortem caspase-3 levels are high. Further studies are needed to investigate the implications of abnormal apoptotic proteins in schizophrenia.

Neuronal apoptosis is highly active during early neurodevelopment. It is estimated that, depending on the region, 20%–80% of all neurons formed in the CNS die by apoptosis in early life (1). Apoptosis is regulated by several protein families, including the upstream Bcl-2 family (e.g., the antiapoptotic Bcl-2 and proapoptotic Bax) and the downstream caspase family (e.g., caspase-3). Pathological activation of neuronal apoptosis and abnormal expression of apoptotic regulatory proteins have been demonstrated in classic neurodegenerative disorders, including Alzheimer’s and Parkinson’s diseases (2–5). Apoptosis has also been hypothesized to contribute to the pathophysiology of schizophrenia (6), a neurodevelopmental disorder that may encompass limited neurodegenerative features (7). Postmortem evidence supports apoptotic involvement in schizophrenia; low levels of Bcl-2 have been demonstrated in Brodmann’s area 21 (middle temporal gyrus) (8). It is interesting that this cortical area is also associated with low gray matter volume (9), similar to low volumes in other temporal and frontal areas and in total cortical gray matter (10, 11). Low levels of Bcl-2 in Brodmann’s area 21 may signal a role for apoptotic proteins in the mechanisms underlying the low gray matter volumes in schizophrenia.

The activation of apoptosis is controlled at multiple checkpoints within the cell. Upstream, the proapoptotic Bax and antiapoptotic Bcl-2 are membrane-bound pore-forming proteins that interact through heterodimerization. Together they regulate the mitochondrial transmembrane passage of cytochrome c, which in turn activates caspase proteins. The Bax/Bcl-2 ratio appears more important than the individual Bax or Bcl-2 level in determining a cell’s vulnerability to apoptosis; high Bax/Bcl-2 ratios lead to greater apoptotic activity (12). Neuronal apoptosis in the CNS is significantly lower than normal in Bax-deleted mice and in Bcl-2-overexpressing mice, while in Bcl-2-deleted mice and in Bax-overexpressing mice, apoptosis is greater than normal (13). Furthermore, in rat cortical development, the Bax/Bcl-2 ratio is highest during the first 2 postnatal weeks, which corresponds to the most active interval of neurodevelopmental apoptosis, while the Bax/Bcl-2 ratio decreases during normal aging, when neuronal apoptosis is rare (13, 14). A high Bax/Bcl-2 ratio has
been demonstrated in the cortex of brains from patients with Down’s syndrome (15) and in human neuronal cultures treated with amyloid β, a model for Alzheimer’s disease (16).

Caspase proteins are cysteine proteases that act downstream of the Bcl-2 family by initiating cellular breakdown during apoptosis (17). Initiator caspases (e.g., caspase-8 and caspase-9) serve to activate effector caspases (e.g., caspase-3, caspase-6, and caspase-7). Among the effector caspases, caspase-3 is most frequently involved in neuronal apoptosis (18). Cleavage of inactive procaspase-3 protein into active caspase-3 subunits is a marker of apoptotic activity (19), and levels of activated caspase-3 subunits are significantly higher than normal in both the cortex of patients with Alzheimer’s disease (4) and the substantia nigra of patients with Parkinson’s disease (5). Thus, measurement of caspase-3 can yield information about ongoing neuronal apoptosis and provide a pathophysiological comparison to classic neurodegeneration.

This is a continuing investigation of apoptotic proteins in the temporal cortex of schizophrenia patients. It has been demonstrated that in these patients the temporal cortex, including Brodmann’s area 21, has a low volume of gray matter (9, 20–22), experiences progressive volume loss in early stages of the illness (23, 24), and is involved in certain clinical manifestations of schizophrenia, including auditory hallucinations (20, 25). Western blot analysis was used to measure Bax, Bcl-2, procaspase-3, and activated caspase-3 subunit levels in blocks of Brodmann’s area 21. The primary hypothesis was that the Bax/Bcl-2 ratio would be high in patients with schizophrenia.

Method

Postmortem Samples

This study was approved by the institutional review board of the University of North Carolina School of Medicine. The postmortem middle temporal gyrus (Brodmann’s area 21) from 60 subjects was obtained from the Stanley Medical Research Institute (Bethesda, Md.); the subjects comprised patients with schizophrenia, bipolar disorder, or major depression and nonpsychiatric comparison subjects (N=15 for each group). Subjects over the age of 68 years were excluded to minimize comorbid neurological disorders. The subjects were group matched for age, gender, ethnicity, side of brain, brain pH, and postmortem interval (Table 1). Demographic and clinical characteristics for individual subjects, including cumulative antipsychotic medication exposure and medications at the time of death, have been extensively detailed elsewhere (8, 26). All samples were stored at –80°C until use. The schizophrenia and nonpsychiatric comparison groups were the primary focus of this study. All data were collected by investigators blind to diagnosis.

Procedure

Tissue homogenization. Tissue (100–300 mg) was placed in 10 volumes of 10 mM HEPES buffer (pH 7.0) with 0.32 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 mM benzamidine, and 0.1 mM benzthionium chloride. The samples were homogenized on ice for 30 seconds and sonicated for 10 seconds at 10 mV. The tissue samples were centrifuged for 15 minutes at 3,000 rpm and 4°C. The supernatants were assayed for total protein by the bicinchoninic acid method (Micro BCA Protein Assay Kit, Pierce Chemical, Rockford, Ill.). All chemicals were obtained from Sigma (St. Louis).

Semiquantitative Western blots. Adapting previously described methods (8), we resolved the samples on 12% (Bcl-2, procaspase-3), 14% (Bax), and 16% (activated caspase-3) Tris-glycine polyacrylamide gels using a mini-cell electrophoresis unit (XCell II, NOVEX, San Diego). Equal amounts of total protein (10 μg for Bax, 30 μg for Bcl-2, 20 μg for procaspase-3, and 90 μg for activated caspase-3) were boiled for 5 minutes in Tris-glycine sodium dodecyl sulfate sample buffer and applied to triplicate gels. All gels were run with a low-range molecular weight ladder (Rainbow MW Marker, Amersham Pharmacia, Piscataway, N.J.) and controls as follows: HeLa cell lysate (Transduction Laboratories, Lexington, Ky.) for Bax and Jurkat cell lysate (Transduction Laboratories) for Bcl-2 and procaspase-3. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, Mass.) at 25 V for 90 minutes, and complete transfer was ascertained with Ponceau S stain. Nonspecific protein binding was blocked for 1 hour with 5% blocking reagent in 0.1% Tween at room temperature. The membranes were incubated for 90 minutes at 25°C with primary antihuman antibodies as follows: rabbit polyclonal Bax (N-20, 1:2000, Santa Cruz Biotechnology, Santa Cruz, Calif.), mouse monoclonal Bcl-2 (1:500, Transduction Laboratories), mouse monoclonal procaspase-3 (CPP32, 1:1000, Transduction Laboratories), mouse monoclonal activated caspase-3 (IMG 144, 1:200, Imgenex, San Diego, Calif.). The membranes were then incubated for 1 hour with appropriate secondary antibodies labeled with horseradish peroxidase, as follows: antimouse antibodies (1:1000) for monoclonal primary antibodies and anti-rabbit antibodies (1:1000) for polyclonal primary antibodies. The membranes were developed using chemiluminescence (ECL, Amersham), and protein bands were detected on radiographic film (Hyperfilm ECL, Amersham). Optical densitometry was performed by using the public domain program NIH Image 1.62 (http://rsb.info.nih.gov/nih-image/), and image analysis was accomplished by means of Adobe Photoshop 5.0 (Adobe Systems, San Jose, Calif.). All raw optical densities were normalized to a pooled control sample applied to all gels to allow for intergel comparisons. In addition, a standard curve of pooled control sample was immunoblotted for each protein to ascertain that all samples fell within the linear portion of the densitometric

<p>| TABLE 1. Characteristics of Postmortem Brain Tissue From Patients With Schizophrenia and Three Comparison Groups |
|-----------------|-------------------------------|-----------------|-----------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>Age (years)</th>
<th>Postmortem Interval (hours)</th>
<th>Brain pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpsychiatric comparison subjects (N=15)</td>
<td>M</td>
<td>F</td>
<td>White</td>
<td>Black</td>
<td>Asian</td>
</tr>
<tr>
<td>Patients with schizophrenia (N=15)</td>
<td>9</td>
<td>6</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Patients with bipolar disorder (N=15)</td>
<td>9</td>
<td>6</td>
<td>13</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Patients with major depression (N=15)</td>
<td>9</td>
<td>6</td>
<td>15</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

a No significant difference among groups (F=1.87, df=3, 59, p=0.15).
b No significant difference among groups (F=0.71, df=3, 59, p=0.55).

http://ajp.psychiatryonline.org
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curvature (data not shown). The Bax/Bcl-2 ratio was determined as follows: 1) the optical densities of Bax and Bcl-2 for each sample were standardized to the mean optical densities of the nonpsychiatric comparison group for Bax and Bcl-2, respectively, then 2) Bax/Bcl-2 quotients of the standardized Bax and Bcl-2 values were calculated for each sample. These indicate the difference in the Bax/Bcl-2 ratio for each sample from the mean of the comparison group.

**Protein Stability and Drug Effects**

**Postmortem stability of Bax and caspase-3.** Sprague-Dawley rats (150–200 g, Charles River Laboratories, Raleigh, N.C.) were killed and separated into two gender-matched groups (N=4 per group). In one group, the frontal cortex was dissected and immediately frozen at −80°C. To approximate the human postmortem condition, the heads of the other group were kept at 25°C for 6 hours and then at 4°C for 18 hours, after which the frontal cortex was dissected and frozen. All tissues were homogenized and sonicated, and Western blotting of Bax and caspase-3 was performed as described in the preceding by using primary antibodies for Bax and caspase-3. Bcl-2 postmortem stability has previously been determined (27).

**Haloperidol effects on Bax, Bcl-2, and caspase-3.** Sprague-Dawley rats (150–200 g) received haloperidol, 1 mg/kg per day (N=7), or saline (N=6) intraperitoneally for 7 days. One hour after the final dose, the rats were killed and the frontal cortex was dissected and homogenized. Western blotting was performed by using primary antibodies for Bax, caspase-3, and Bcl-2 as described in the preceding.

**Statistical Analysis**

Statistical analysis was performed by using GraphPad Prism, version 3.00 (GraphPad Software, San Diego, Calif.). A priori, primary analysis was limited to the specimens of the nonpsychiatric comparison subjects versus those of the schizophrenia patients, given our hypothesis. The Bax/Bcl-2 ratio was the primary outcome variable, and the two groups were compared by using a Student’s t test with two-tailed p values considered significant at p<0.05. Secondary analyses included individual comparisons of Bax, Bcl-2, and caspase-3 levels (active and zymogen forms) by Student’s t tests in the schizophrenia and comparison groups. Apoptotic protein levels were also compared by means of one-way analysis of variance (ANOVA) across all diagnostic groups. The Bax/Bcl-2 ratio and activated caspase-3 subunit levels were analyzed for age, gender, side of brain, and antipsychotic medication effects. In the rat studies, postmortem stability and haloperidol effects were analyzed by using Student’s t tests.

**Results**

**Bax and Bcl-2 Immunoblots**

The mean Bax/Bcl-2 ratio was 50% higher in the schizophrenia group (mean=1.65, SD=0.90) than in the nonpsychiatric comparison group (mean=1.10, SD=0.37) (Figure 1 and Figure 2). Individually, the mean Bax level in the patients with schizophrenia (mean=1.18 normalized optical density, SD=0.59) did not differ from that of the comparison subjects (mean=1.06, SD=0.25) (t=0.72, df=28, p=0.46) (Figure 2), and the mean Bcl-2 level of the schizophrenia group (mean=1.39, SD=0.75) did not differ from that of the comparison group (mean=1.75, SD=0.69) (t=1.35, df=28, p=0.19) (Figure 2). It was the effect of differences in the Bax and Bcl-2 levels and the resulting Bax/Bcl-2 ratio for each individual subject that produced a substantial difference in Bax/Bcl-2 ratio between the two groups.

There was a nearly significant group effect on the mean Bax/Bcl-2 ratio across all psychiatric groups according to ANOVA (F=2.36, df=3, 59, p=0.09). The mean ratios for the patients with bipolar disorder (mean=1.18, SD=0.71) and major depression (mean=1.12, SD=0.52) were close to that of the nonpsychiatric comparison subjects (mean=1.10, SD=0.37). The ratio did not correlate with age across all groups (r²=0.01, N=60, p=0.53) or with lifetime fluoxetine-equivalent dose in the schizophrenia group (r²=0.16, N=15, p=0.14). The Bax/Bcl-2 ratio also did not differ between women (mean=1.11, SD=0.53) and men (mean=1.09, SD=0.52) in the schizophrenia group. There was a nearly significant group effect on the mean Bax/Bcl-2 ratio across all psychiatric groups according to ANOVA (F=2.36, df=3, 59, p=0.09). The mean ratios for the patients with bipolar disorder (mean=1.18, SD=0.71) and major depression (mean=1.12, SD=0.52) were close to that of the nonpsychiatric comparison subjects (mean=1.10, SD=0.37). The ratio did not correlate with age across all groups (r²=0.01, N=60, p=0.53) or with lifetime fluoxetine-equivalent dose in the schizophrenia group (r²=0.16, N=15, p=0.14). The Bax/Bcl-2 ratio also did not differ between women (mean=1.11, SD=0.53) and men (mean=1.09, SD=0.52) in the schizophrenia group.
Caspase-3 Immunoblots

The immunoblots exposed to activated caspase-3 antibodies revealed two large-fragment bands migrating to 20 and 17 kDa (Figure 1). Three isoforms of the large caspase-3 subunit have previously been described at 20, 19, and 17 kDa (28). Among these bands, the 20-kDa isoform was most readily quantifiable on our gels, and it was 8% lower in the schizophrenia group (mean=1.01 normalized optical density, SD=0.09) than in the nonpsychiatric comparison group (mean=1.10, SD=0.16) (t=2.00, df=27, p=0.06) (Figure 3). The small caspase-3 subunit migrating to 12–14 kDa could not be measured reliably across all samples owing to faint expression and blot artifacts, but no qualitative group differences were evident (Figure 1). Although the difference was not quite significant, the procaspase-3 levels also appeared somewhat lower in the schizophrenia group (mean=0.87 normalized optical density, SD=0.33) than in the nonpsychiatric comparison group (mean=1.08, SD=0.29) (t=1.87, df=28, p=0.08) (Figure 1 and Figure 3).

Postmortem Stability of Bax and Caspase-3

The Western blots of rat cortex revealed high postmortem stability of the Bax and caspase-3 proteins over 24 hours. The mean Bax level in the rat cortex 24 hours postmortem (mean=0.20 optical density, SD=0.02) was not significantly different from that at 0 hours postmortem (mean=0.22, SD=0.02) (t=1.79, df=6, p=0.13). Likewise, the mean value for the caspase-3 20-kDa subunit at 24 hours postmortem (mean=0.22 optical density, SD=0.00) was unchanged from that at 0 hours (mean=0.22, SD=0.00) (t=1.10, df=5, p=0.33).

Haloperidol Effects on Bax, Bcl-2, and Caspase-3

The mean Bax/Bcl-2 ratio in the frontal cortex of the haloperidol-treated rats (mean=0.88, SD=0.12) was not significantly different from that of the rats given the saline control (mean=0.99, SD=0.18) (t=1.47, df=11, p=0.17). There was also no significant difference in the mean Bax level between haloperidol (mean=0.99 normalized optical density, SD=0.10) and saline (mean=1.10, SD=0.23) (t=1.14, df=11, p=0.28) or in the mean Bcl-2 level between haloperidol (mean=0.89, SD=0.06) and saline (mean=0.86, SD=0.06) (t=1.01, df=11, p=0.34). The mean level for the 20-kDa caspase-3 subunit also did not differ between haloperidol (mean=1.01 normalized optical density, SD=0.28) and saline (mean=1.04, SD=0.31) (t=0.21, df=11, p=0.85).
Discussion

In this study, the mean Bax/Bcl-2 ratio in Brodmann’s area 21 was 50% higher in patients with schizophrenia than in nonpsychiatric comparison subjects. This finding provides new evidence that apoptotic proteins contribute to the underlying pathophysiology of schizophrenia. A high Bax/Bcl-2 ratio has been found to increase the vulnerability of neurons to apoptosis (12), and a high ratio occurs in several classic neurodegenerative disorders characterized by progressive neuronal loss (15, 16). In schizophrenia, this finding may be particularly relevant to the evidence for progressive gray matter loss, as seen most consistently in early stages of the disorder (22–24, 29, 30).

The individual mean levels of Bax and Bcl-2 in the schizophrenia patients did not differ from those of the comparison subjects. Using ELISA, we previously detected 25% less Bcl-2 in schizophrenia patients than comparison subjects in Brodmann’s area 21 (8). Numerically, the Bcl-2 level was 21% lower in the schizophrenia patients in the current study, but the lack of significance probably reflects the lower sensitivity of Western blot compared to ELISA. It was necessary to reanalyze Bcl-2 with a different method—Western blot, in triplicate—in the current study in order to derive the Bax/Bcl-2 ratio since a Bax ELISA was unavailable. Furthermore, Western blot has the advantage of quantification and protein identification within one technique, while ELISA does not allow direct visualization of protein bands.

In contrast to the high Bax/Bcl-2 ratio, neither the activated caspase-3 nor procaspase-3 level was statistically different from that of the nonpsychiatric comparison subjects, although they both were modestly lower. This result may indicate that apoptosis is not active in Brodmann’s area 21 in chronic schizophrenia. However, this interpretation is qualified by several considerations. First, apoptosis may be active only during distinct phases of illness. For example, if apoptotic activity is limited to prodromal and new-onset schizophrenia, when progressive gray matter loss has been identified, then the current study using tissue from patients with chronic illness would miss such activity. Second, a high Bax/Bcl-2 ratio may reduce neuronal viability in the absence of caspase-3 activity and/or involve other effector caspases. It is interesting that the somewhat lower caspase-3 levels may have relevance to a recent study demonstrating a subset of neurons with fewer single-stranded DNA breaks in the cingulate cortex of patients with schizophrenia (31). In contrast, consistently higher procaspase-3 and activated caspase-3 levels in Alzheimer’s and Parkinson’s diseases (4, 5) suggest that the pathophysiology of schizophrenia is distinct from classic neurodegeneration.

The current findings raise several other considerations for the pathophysiology of schizophrenia. First, schizophrenia is increasingly conceptualized as a disorder of the synapse, given converging lines of evidence from microarray, neuropathological, and neuroimaging techniques (32, 33). Furthermore, several studies suggest that synaptic remodeling and elimination may be influenced by apoptotic protein activity in a process termed “synaptic apoptosis” (34, 35). Although this process is speculative, if synaptic loss in schizophrenia is mediated by apoptotic protein activity and is episodic rather than continuous (e.g., during adolescent synaptic pruning or in response to discrete episodes of excitotoxic stress), then the caspase-3 level may be increased only transiently and then return to baseline. Second, the data do not rule out an early neurodevelopmental role for apoptosis in schizophrenia. A fetal or perinatal insult could transiently alter normal developmental apoptosis to yield enduring cytoarchitectural deficits. It is
interesting that multiple proapoptotic stimuli, such as ischemia, hypoxia, and pro-inflammatory cytokines (36, 37), have also been implicated as insults during early life that increase the risk of developing schizophrenia (38–40). Studies of the normal developmental pattern of apoptotic protein expression in the human frontal cortex have revealed that infancy is the period of highest Bax levels and lowest Bcl-2 levels across the lifespan (27, 41). This suggests that neuronal apoptosis during the perinatal period may be especially vulnerable to dysregulation from neurodevelopmental insults.

All of the subjects in this study were group matched for age, gender, postmortem interval, side of brain, and brain pH, limiting the potential confounding effects of these variables. Although chronic antipsychotic treatment may alter the expression of apoptotic proteins, one week of haloperidol treatment in animals did not produce changes. Furthermore, cumulative antipsychotic medication exposure did not correlate with the Bax/Bcl-2 ratio or caspase-3 level. Postmortem interval could affect results if apoptotic proteins demonstrated poor postmortem stability. However, the study of postmortem stability showed no change in Bax, procaspase-3, or activated caspase-3 (20-kDa subunit) over 24 hours. Similarly, high postmortem stability for Bcl-2 was previously determined (27). Finally, patient-related factors, including diagnostic heterogeneity, substance abuse, and medical illness, could introduce unintended variability, but efforts were made to limit these factors during subject selection (26).

This study revealed limited associations with other psychiatric diagnoses. The Bax/Bcl-2 ratio and caspase-3 level were essentially no different in the patients with bipolar disorder or major depression from those in the nonpsychiatric comparison subjects. Since substantially lower cortical volume and neuronal and glial density have been documented in affective disorders (42, 43), this finding suggests that divergent pathophysiological mechanisms may underlie the neuropathology of schizophrenia and primary affective disorders.

In conclusion, this study indicates that apoptotic proteins may reduce neuronal viability in adult schizophrenia without inducing large-scale apoptosis. An intriguing possibility that merits further inquiry is the extent to which apoptotic proteins influence synaptic elimination and thereby contribute to neuropil loss in schizophrenia. The differential regulation of the expression of Bax, Bcl-2, and caspase-3 proteins demonstrates that the pathophysiology of schizophrenia is distinct from that of classic neurodegenerative disorders. Ultimately, if clinically relevant roles for apoptotic proteins can be demonstrated, their pharmacological manipulation may permit novel approaches to schizophrenia treatment.

References

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